

Differential expression of prostaglandin (PG) synthesis enzymes in conceptus during peri-implantation period and endometrial expression of carbonyl reductase/PG 9-ketoreductase in the pig

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Abstract

Prostaglandins (PGs) play a pivotal role in luteolysis, maternal recognition of pregnancy, and implantation. In many species, including pigs, both conceptus (embryo and associated membranes) and endometrium synthesize PGE₂, which may antagonize PGF_{2α} by playing a luteotropic/antiluteolytic role. Previously, we have reported expression profiles of PG G/H synthases (PGHS-1 and PGHS-2), PGE synthase (mPGES-1), and PGF synthase (PGFS) in the endometrium of cyclic and pregnant pigs. In the present study, expression of above-mentioned PG synthesis enzymes and PG 9-ketoreductase (CBR1), which converts PGE₂ into PGF_{2α}, and the PGE₂/PGF_{2α} ratios were investigated in porcine peri- and post-implantation conceptuses. Furthermore, expression of CBR1 was examined in the endometrium. PGHS-2 and mPGES-1 were upregulated, and PGHS-1, PGFS, and CBR1 were downregulated in conceptuses during trophoblastic elongation. A second increase of mPGES-1 mRNA occurred

after days 20–21 of pregnancy. After initiation of implantation, expression of PGHS-1, PGFS, and CBR1 in conceptuses increased and remained higher until days 24–25 of pregnancy. Comparison of the endometrial CBR1 protein expression in cyclic and pregnant gilts revealed upregulation on days 16–17 of the cycle and downregulation on days 10–11 of pregnancy. In conclusion, reciprocal expression of PGHS-2, mPGES-1, PGFS, and CBR1 in day 10–13 conceptuses and decrease of endometrial CBR1 may be important in increasing the PGE₂/PGF_{2α} ratio during maternal recognition of pregnancy. This study indicates that PGE₂ produced via PGHS-2 and mPGES-1 in conceptus may be involved in corpus luteum control. Moreover, high expression of conceptus PGHS-1, mPGES-1, PGFS, and CBR1 after initiation of implantation suggests their significant role in placentation.

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Introduction

Maternal recognition of pregnancy requires reciprocal communication between the pre-implantation conceptus (embryo and associated membranes) and maternal system: uterine endometrium and corpus luteum (CL). In the pig, these interactions begin at days 11–12 of pregnancy when conceptuses undergo rapid elongation (Geisert *et al.* 1982a) and signal their presence to maternal system by estrogen secretion (Perry *et al.* 1973, Bazer & Thatcher 1977, Ford *et al.* 1982). However, estrogen treatment alone, although preventing prostaglandin F_{2α} (PGF_{2α})-induced luteal regression and prolonging CL lifespan, cannot fully mimic the conceptus effects on growth and development of this gland (Christenson & Ford 1995) as well as an endocrine status in the pig (Ziecik *et al.* 1986). It has been suggested that besides estrogens, PGE₂ could be involved in maternal recognition of pregnancy as a luteoprotective/antiluteolytic factor (Akinlosotu *et al.* 1986, Christenson *et al.* 1994). The increased secretion of PGE₂ by the gravid versus the

non-gravid horn of the uterus is associated with an elevated progesterone concentration of CL on the adjacent ovary in the pig (Christenson *et al.* 1994). Moreover, on days 11–13 of pregnancy, a dramatic increase of PGE₂/PGF_{2α} ratio is observed in uterine lumen and vein when compared with the respective days of the estrous cycle in the pig, suggesting that PGE₂ can overcome luteolytic effect of PGF_{2α}. Since PGE₂ and PGF_{2α} exert mainly opposite actions, the PGE₂/PGF_{2α} ratio may be an important mediator or modulator of several processes in female reproduction (Weems *et al.* 2006). The PGE₂/PGF_{2α} ratio has an influence on CL development and function, endometrial cell growth and differentiation, myometrium contraction, blood flow, vascular permeability, embryo migration, and implantation (Davis & Blair 1993, Cao *et al.* 2005).

Not only the endometrium but also blastocysts from many species, including mouse (Marshburn *et al.* 1990), rat (Parr *et al.* 1988), rabbit (Dey *et al.* 1980), sheep (Hyland *et al.* 1982), cow (Lewis *et al.* 1982), human (Holmes & Gordashko 1980), and pig (Davis *et al.* 1983) appear to have the capacity

to transform arachidonic acid into its biological active derivatives via PG G/H synthase enzyme (PGHS) pathway, resulting in the production of PGE₂ and PGF_{2α}. The mechanism responsible for maintenance of CL during early pregnancy is complex and may involve a specific expression pattern of PG synthesis enzymes in conceptus and the direct influence of the embryo on endometrial PG synthesis. PGHS converts arachidonic acid into PGH₂, which is further metabolized to PGs by specific synthases and reductases (Smith *et al.* 1996). There are two isoforms of PGHS known: PGHS-1 and PGHS-2 whose roles in reproduction have been established (Sales & Jabbour 2003, Murakami & Kudo 2004). PGHS-1 is considered to have mainly constitutive expression in many tissues, while PGHS-2 appears to be an inducible form. Nevertheless, limited information is available on the PGHS-2 expression in blastocysts in the pig (Wilson *et al.* 2002). Moreover, nothing is known about regulation of expression of downstream enzymes: PGE synthase (mPGES-1), PGF synthase (PGFS), and PG 9-ketoreductase in conceptus development. Although changes of PG concentration in porcine uterine lumen are well characterized in the estrous cycle and early pregnancy, content of PGs was studied only in porcine day 7–12 embryos (Davis *et al.* 1983).

We have recently shown expression patterns of PGHS-1, PGHS-2, mPGES-1, and PGFS in the endometrium of porcine species (Blitek *et al.* 2006, Waclawik *et al.* 2006). However, expression of an important enzyme catalyzing NADP(H)-dependent reversible conversion of PGE₂ into PGF_{2α}, PG 9-ketoreductase is unknown. Carbonyl reductase (CBR1), also known as 20β-hydroxysteroid dehydrogenase is considered to be identical with PG 9-ketoreductase in the pig (Schieber *et al.* 1992, Tanaka *et al.* 1992, Ghosh *et al.* 2001). The ability of CBR1 to reduce a large number of biological and pharmacological carbonyl compounds has complicated the systematic nomenclature of the enzyme. Three different numbers (EC. 1.1.1.184, 1.1.1.189, and 1.1.1.197) were originally assigned to the enzyme (Maser 1995). Sequence analysis reveals that porcine CBR1 belongs to the short-chain dehydrogenase/reductase superfamily (Jornvall *et al.* 1981, Tanaka *et al.* 1992).

Interestingly, expression of PG 9-ketoreductase gene is downregulated in epithelial cells of bovine endometrium by interferon-τ, which is the conceptus signal in ruminants (Asselin & Fortier 2000). These findings suggest that conceptus can alter expression of endometrial PG 9-ketoreductase (CBR1) in order to modulate the PGE₂/PGF_{2α} ratio in the uterus during the maternal recognition of pregnancy. Therefore, the objectives of the present study were to 1) characterize steady state levels of mRNA and proteins of PG biosynthesis enzymes (PGHS-1, PGHS-2, mPGES-1, PGFS, and CBR1) in porcine conceptuses during early pregnancy in the pig; 2) determine the ratios of PGE₂ to PGF_{2α} and 13,14-dihydro-15-keto-PGF_{2α}, the major stable metabolite of PGF_{2α} (PGFM) to PGF_{2α} in the peri-implantation conceptuses; and 3) evaluate the changes of endometrial expression of CBR1.

Materials and Methods

Tissue collection

The endometrium was collected from 45 cyclic crossbred gilts (Large White × Polish Landrace) at a local abattoir. The stage of the estrous cycle was defined by utero-ovarian morphology (Akins & Morissette 1968, Leiser *et al.* 1988). Moreover, the histological features of the uterus and CL were used to verify the stage of estrous cycle, as reported previously (Leiser *et al.* 1988). The endometrium dissected from myometrium was recovered from the middle portion of uterine horn and was accordingly assigned to the following days of the estrous cycle: 1–4 (*n* = 7), 5–9 (*n* = 8), 10–11 (*n* = 6), 12–13 (*n* = 5), 14–15 (*n* = 8), 16–17 (*n* = 5), 18–19 (*n* = 3), and 20–21 (*n* = 3).

Furthermore, 57 gilts after exhibiting two estrous cycles of normal length were bred 12 and 24 h after the onset (day 0) of estrus. Pregnant gilts were slaughtered at a local abattoir on days 5–9 (*n* = 5), 10–11 (*n* = 12), 12–13 (*n* = 5), 14–15 (*n* = 6), 16–17 (*n* = 8), 18–19 (*n* = 6), 20–21 (*n* = 5), 22–23 (*n* = 4), and 24–25 (*n* = 6) of pregnancy, and endometrium was collected from the uterus opened longitudinally on the antimesometrial surface. Pregnancy was confirmed by the presence of conceptuses. During the pre-implantation stage, both uterine horns were flushed with 20 ml sterile PBS to recover conceptuses. During implantation and early placentation stage, conceptuses/trophoblasts were dissected from endometrium. Dissection of trophoblast tissues from embryo was done only after days 17–18 post-mating; therefore, expressions of the studied enzymes were analyzed in 10- to 17-day conceptuses and 18- to 25-day trophoblast tissues. Furthermore, endometrial tissue was collected from implantation sites by dissection from myometrium.

Based on the days of pregnancy and conceptus morphology, the conceptuses or trophoblast tissues derived from the same animal were pooled and classified into groups as days 10–13 (spherical and tubular, *n* = 4), days 10–13 (filamentous, *n* = 4), 14–15 (*n* = 5), 16–17 (*n* = 5), 18–19 (*n* = 6), 20–21 (*n* = 3), 22–23 (*n* = 4), and 24–25 (*n* = 7). Conceptuses pooled from each uterus separately revealed similar morphology (spherical/tubular or filamentous).

Conceptus/trophoblast, endometrial, and other tissue samples (liver, kidney, lung, CL, 20-day embryo, oviduct, brain, heart, and myometrium) were cut into small pieces and snap-frozen in liquid nitrogen and stored at –80 °C until further use. 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.

Total RNA isolation

Total RNA was extracted from endometrial and conceptus/trophoblast samples using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987) and treated with DNase I (Invitrogen Life Technology Inc.)

according to the manufacturer's protocol to eliminate possible DNA contamination.

Real-time PCR quantitation

Real-time PCR was performed with the Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR master mix (Qiagen GmbH), as described previously (Waclawik *et al.* 2006). Briefly, total RNA was reverse transcribed using oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR mix (50 μ l) included 25 μ l QuantiTect SYBR Green PCR master mix, 0.5 μ M sense and antisense primers each and reverse-transcribed cDNA (3 μ l diluted RT product). To evaluate mRNA levels of the enzymes, specific primers were used (Table 1). 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: 38 cycles of denaturation (15 s at 95 °C), annealing (30 s at 52.5 °C for PGFS and CBR1; or at 55 °C for PGHS-1, PGHS-2, mPGES-1, and β -actin), and elongation (60 s at 72 °C). After PCR, melting curves were acquired by stepwise increases in the temperature from 50 to 95 °C to ensure that a single product was amplified in the reaction. On the basis of our preliminary data and previous reports (Yelich *et al.* 1997, Madore *et al.* 2003, Blomberg *et al.* 2005, Waclawik *et al.* 2006), β -actin was used as an internal control for normalization of the real-time PCR data for PGHS-1, PGHS-2, mPGES-1, PGFS, and CBR1 expression in the cyclic and pregnant endometrium and peri-implantation conceptuses. Control reactions in the absence of reverse transcriptase were performed to test for genomic DNA contamination. Furthermore, specificity of RT-PCR products was confirmed by sequencing. The sequences were compared against PGHS-

1, PGHS-2, mPGES-1, PGFS, CBR-1, and β -actin cDNAs (GenBank accession numbers indicated in Table 1).

Preparation of cytosol and membrane fractions for western blot

Protein fractions for immunoblotting were obtained as described previously (Waclawik *et al.* 2006). Briefly, endometrial, conceptus/trophoblasts, and other tissues were homogenized on ice in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10 μ g/ml aprotinin, 52 μ M leupeptin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Homogenates were then centrifuged for 10 min at 1000 g at 4 °C. The supernatant was centrifuged for 1 h at 105 000 g at 8 °C and the resulting supernatant and precipitate were used as the cytosol and membrane fraction respectively. The fractions were stored at -70 °C for further analysis. The protein concentration was determined by the Bradford (1976) method.

Western blot analysis

Equal amounts (30 μ g protein) of membrane (for mPGES-1) and cytosol fractions (for PGFS and CBR1) were dissolved in SDS gel-loading buffer (50 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 2% (v/v) β -mercaptoethanol), heated to 95 °C for 4 min, and separated on 15% (w/v; for mPGES-1) and 12% (w/v; for PGFS and CBR1) 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, and 20% (v/v) methanol). After blocking in 5% (w/v) non-fat dry milk in TBS-T buffer (Tris-buffered saline, containing 0.1% (v/v) Tween 20) for 1.5 h at 25.6 °C, the membranes were incubated overnight with 1:1000 polyclonal anti-mPGES-1 antibodies (Cayman Chemical, Ann Arbor, MI, USA), 1:2000 anti-lung-type PGFS antiserum (Watanabe *et al.* 1985), or 1:2000 polyclonal anti-human CBR1 antibodies (Abcam, Cambridge, UK), or

Table 1 Primers used for real-time PCR

Gene	Primer sequences	GenBank accession number	Reference
PGHS-1	Sense: 5'-GGGAGTCCTTCTCCAATGTG-3' Antisense: 5'-CATAAATGTGGCCGAGGTCT-3'	AF207823	Blitek <i>et al.</i> (2006)
PGHS-2	Sense: 5'-ATGATCTACCCGCTCACAC-3' Antisense: 5'-AAAAGCAGCTCTGGGTCAA-3'	AY028583	Blitek <i>et al.</i> (2006)
mPGES-1	Sense: 5'-ATCAAGATGTACGTAGTGGC-3' Antisense: 5'-GAGCTGGGCCAGGGTGTAGG-3'	AY857634	Waclawik <i>et al.</i> (2006)
PGFS	Sense: 5'-GGACTTGGCACTCTCGTCTC-3' Antisense: 5'-GAACAGCTCCTCCCTTCA-3'	AY863054	Waclawik <i>et al.</i> (2006)
CBR1	Sense: 5'-CTTCCACCAGCTGGACATC-3' Antisense: 5'-CATTCAAGGAGGATCTGTCC-3'	M80709	
β -actin	Sense: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' Antisense: 5'-GAGGGGCGATGATCTTGATCTCA-3'	U07786	Spagnuolo-Weaver <i>et al.</i> (1999) and Waclawik <i>et al.</i> (2006)

polyclonal anti- β -actin antibodies (Abcam; 1:4000) at 4 °C. Fragments of amino acid sequence of porcine PG 9-ketoreductase, reported earlier, revealed 90% of homology with human CBR1 (Schieber *et al.* 1992). From comparison of several properties (catalytical, structural, and immunological), it is concluded that PG 9-ketoreductase and CBR1 are identical enzymes. Therefore, we used anti-human CBR1 antibodies to detect PG 9-ketoreductase in the pig. Subsequently, the studied enzymes were detected by incubating the membrane with 1:20 000 dilution of secondary polyclonal anti-rabbit alkaline phosphatase-conjugated antibodies (for mPGES-1, PGFS, and β -actin; Sigma–Aldrich) and anti-goat alkaline phosphatase-conjugated antibodies (1:2000, for CBR1; Abcam) for 1.5 h at 25.6 °C. Immune complexes were visualized using standard alkaline phosphatase visualization procedure (Sambrook *et al.* 1989). Western blots were quantitated using Kodak 1D program (Eastman Kodak). β -Actin was used as an internal control for protein loading. To test the specificity of CBR1 antiserum, primary antibodies (1 μ g) were incubated with a specific control peptide (15 μ g; Abcam) in TBS–T buffer for 24 h at 4 °C with gentle shaking. Then, the antibodies blocked by the control peptide were used in western blot analysis.

EIA of PGE₂, PGF_{2 α} , and PGFM

To measure the concentration of PGE₂, PGF_{2 α} , and PGFM, conceptus and trophoblast homogenates were prepared and extracted with ethyl acetate by the method published earlier (Davis *et al.* 1983). The conceptuses were pooled from every uterus separately as described previously. Concentration of PG was expressed per protein content. Concentrations of PGE₂ were determined by an enzyme immunoassay (EIA) as described by Skarzynski & Okuda (2000). Cross-reactivities of the anti-PGE₂ antiserum (donated by Dr Seiji Ito, Kansai Medical University, Osaka, Japan) were as follows: 18% PGE₁, 10% PGA₁, 4.6% PGA₂, 6.7% PGB₂, 0.13% PGD₂, 2.8% PGF_{2 α} , 14% PGJ₂, and 0.05% 15-keto-PGE₂. Assay sensitivity was 0.19 ng/ml and the intra- and inter-assay coefficients of variation were 7.6 and 14.9% respectively. Concentrations of PGF_{2 α} were determined by EIA as described by Uenoyama *et al.* (1997). Cross-reactivities of the anti-PGF_{2 α} antiserum (Sigma–Aldrich) were as follows: 60% PGF_{1 α} ; <0.1% PGE₁ and PGE₂; and <0.01% PGA₁, PGA₂, PGB₁, and PGB₂. Assay sensitivity was 0.23 ng/ml and the intra- and inter-assay coefficients of variation were 7.5 and 11.4% respectively. Concentrations of PGFM were determined by EIA as previously described for PGE₂ (Skarzynski & Okuda 2000) using horseradish peroxidase-labeled PGFM and anti-PGFM antiserum (WS4468-7; donated by Dr William Silvia, University of Kentucky, Lexington, KY, USA). Cross-reactivities of the anti-PGFM antiserum with PGE₂, PGA₂, PGF_{2 α} , and 6-keto-PGF₁ were <0.1%. Assay sensitivity was 50 pg/ml and the intra- and

inter-assay coefficients of variation were 6.2 and 10.1% respectively.

Statistical analysis

Least-squares factorial ANOVA using the general linear models (GLMs) was used to study endometrial CBR1 expression in cyclic and pregnant gilts. These analyses included the effects of reproductive status, day and status \times day interaction. Other statistical analyses were performed using ANOVA, followed by Tukey's multiple comparison test. All numerical data are presented as the mean \pm s.e.m. and differences were considered as statistically significant when $P < 0.05$. Statistical analyses were performed on SPSS 12.0S software for Windows (SPSS Inc., Chicago, USA) or by Graphpad Prism 4.0 (Graphpad Software Inc., San Diego, CA, USA).

Results

Expression of PGHS-1 in conceptus during early pregnancy

PGHS-1 mRNA levels were changing in conceptus during pre-implantation (days 10–13), implantation (days 14–19), and post-implantation period (days 20–25 of pregnancy). PGHS-1 transcript levels were the lowest in spherical/tubular and filamentous conceptuses collected from day 10 to day 13 of pregnancy during pre-implantation period ($P < 0.01$). After initiation of implantation, on days 14–15 of gestation PGHS-1 mRNA levels in conceptus/trophoblast increased \sim 15-fold and remained enhanced to post-implantation period (Fig. 1A).

Expression of PGHS-2 in conceptus during early pregnancy

PGHS-2 transcript content was elevated in filamentous day 10–13 blastocysts when compared with post-elongation conceptuses (days 14–17) and trophoblast tissues from day 22 to day 25 ($P < 0.05$; Fig. 1B). PGHS-2 mRNA levels in filamentous days 10–13 blastocysts were approximately three- to fourfold higher when compared with moderate content in spherical/tubular 10- to 13-day conceptuses and low content in elongated day 14–17 conceptuses ($P < 0.05$). After decrease of PGHS-2 mRNA levels at the beginning of implantation (on days 14–15 of pregnancy), the studied transcript remained at comparable levels until post-implantation period.

Expression of mPGES-1 in conceptus during early pregnancy

Expression of mPGES-1 was highly modulated during conceptus development (Fig. 2A and B). PGE synthase transcript levels were significantly elevated (28-fold times higher than on days 14–15) in spherical/tubular and filamentous day 10–13 conceptuses during pre-implantation period (versus days 14–23, $P < 0.001$; and spherical/tubular

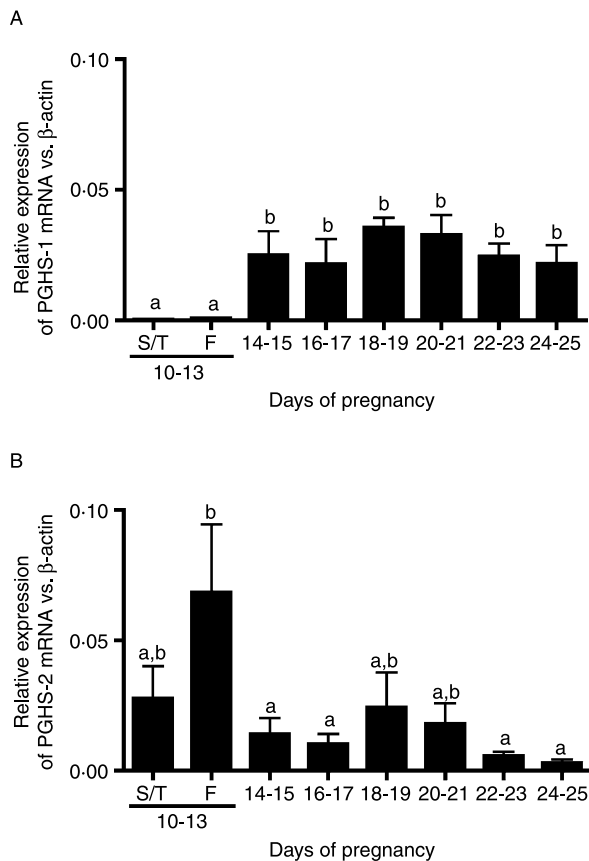


Figure 1 Expression of PGHS-1 (A) and PGHS-2 mRNA (B) in porcine conceptus/trophoblast during early pregnancy. Quantification of prostaglandin G/H synthase mRNA was done by real-time PCR and expressed as the mean \pm S.E.M. of ratios relative to β -actin. For each group, conceptuses/trophoblasts collected from three to seven uteri were analyzed. Conceptuses collected from day 10 to day 13 of pregnancy were divided into two groups according to morphology: spherical, tubular (S/T), and filamentous (F). Means with different letters indicate significant differences ($P < 0.05$).

conceptuses from day 10 to day 13 versus day 24 to day 25; $P < 0.05$). After initiation of implantation, mPGES-1 mRNA content dramatically declined in elongated day 14–17 conceptuses. After days 18–19 of pregnancy, gradual increase of the enzyme mRNA with maximum on days 24–25 was observed (versus days 14–17, $P < 0.001$). Correspondingly, mPGES-1 protein levels were significantly higher on days 10–13 ($P < 0.01$) and days 24–25 ($P < 0.05$) when compared with days 14–21 and intermediate on days 22–23 of pregnancy.

Expression of PGFS in conceptus during early pregnancy

Both PGFS mRNA and protein levels were the lowest in spherical/tubular and filamentous day 10–13 conceptuses ($P < 0.001$ and $P < 0.01$ respectively) during pre-implantation period (Fig. 2C and D). The expression of PGFS mRNA and protein in 10- to 13-day conceptuses was 12- to

24- and 5- to 12-fold lower in comparison with 14- to 25-day conceptuses/trophoblasts respectively. After days 14–15 of pregnancy, PGFS mRNA and protein levels in conceptuses/trophoblasts increased and remained higher during implantation and post-implantation period.

Expression of CBR1 in conceptus during early pregnancy

Patterns of both CBR1 mRNA and protein in conceptus/trophoblasts were parallel (Fig. 2E and F). Content of CBR1 mRNA and protein in spherical/tubular and filamentous conceptuses collected on days 10–13 post-mating, during pre-implantation period was very low ($P < 0.001$) and undetectable respectively. After initiation of implantation, expression of CBR1 mRNA and protein increased and remained high until post-implantation period. Blockage of CBR1 antibodies with the control peptide led to the complete disappearance of bands when compared with antibodies without the blocking peptide (Fig. 4B).

The PGE_2 / $PGF_{2\alpha}$ and $PGFM$ / PGF_2 ratios in conceptus during early pregnancy

PGE_2 concentration was higher ($P < 0.05$) in spherical/tubular and filamentous day 10–13 conceptuses (25.4 and 28.1 ng/mg protein respectively) when compared with conceptuses collected from day 14 to day 19 (5.5 ng/mg protein) and day 24 to day 25 of pregnancy (4.3 ng/mg protein; Table 2). Concentration of PGE_2 increased twofold from implantation to post-implantation period (days 20–23 of pregnancy). $PGF_{2\alpha}$ and $PGFM$ concentration in conceptuses during pre-implantation, implantation, and post-implantation period ranged from 5.4 to 16.5 and 1.3 to 10.2 ng/mg protein respectively, but revealed no significant variation (Table 2).

The paired data analyzed as ratios of PGE_2 to $PGF_{2\alpha}$ and $PGFM$ to $PGF_{2\alpha}$ were determined in conceptus and trophoblast tissues during peri-implantation period (Fig. 3). The PGE_2 / $PGF_{2\alpha}$ ratio was higher ($P < 0.05$) in spherical/tubular day 10–13 conceptuses during pre-implantation stage when compared with days 16–19 and intermediate on days 22–25 of pregnancy (Fig. 3A). The $PGFM$ / $PGF_{2\alpha}$ ratio which is index of 15-hydroxyprostaglandin dehydrogenase activity was significantly lower in spherical/tubular and filamentous 10- to 13-day conceptuses when compared with conceptuses collected on days 22–23 of pregnancy ($P < 0.05$) and days 16–23 ($P < 0.05$) respectively (Fig. 3B). On days 24–25 of pregnancy, the $PGFM$ / $PGF_{2\alpha}$ ratio was decreased when compared with days 16–23 ($P < 0.05$).

CBR1 protein expression in different porcine tissues

CBR1 band of ~ 31.5 kDa was present in all examined tissues (Fig. 4A). The abundance of CBR1 protein was the highest in liver, kidney, and oviduct, and intermediate in myometrium,

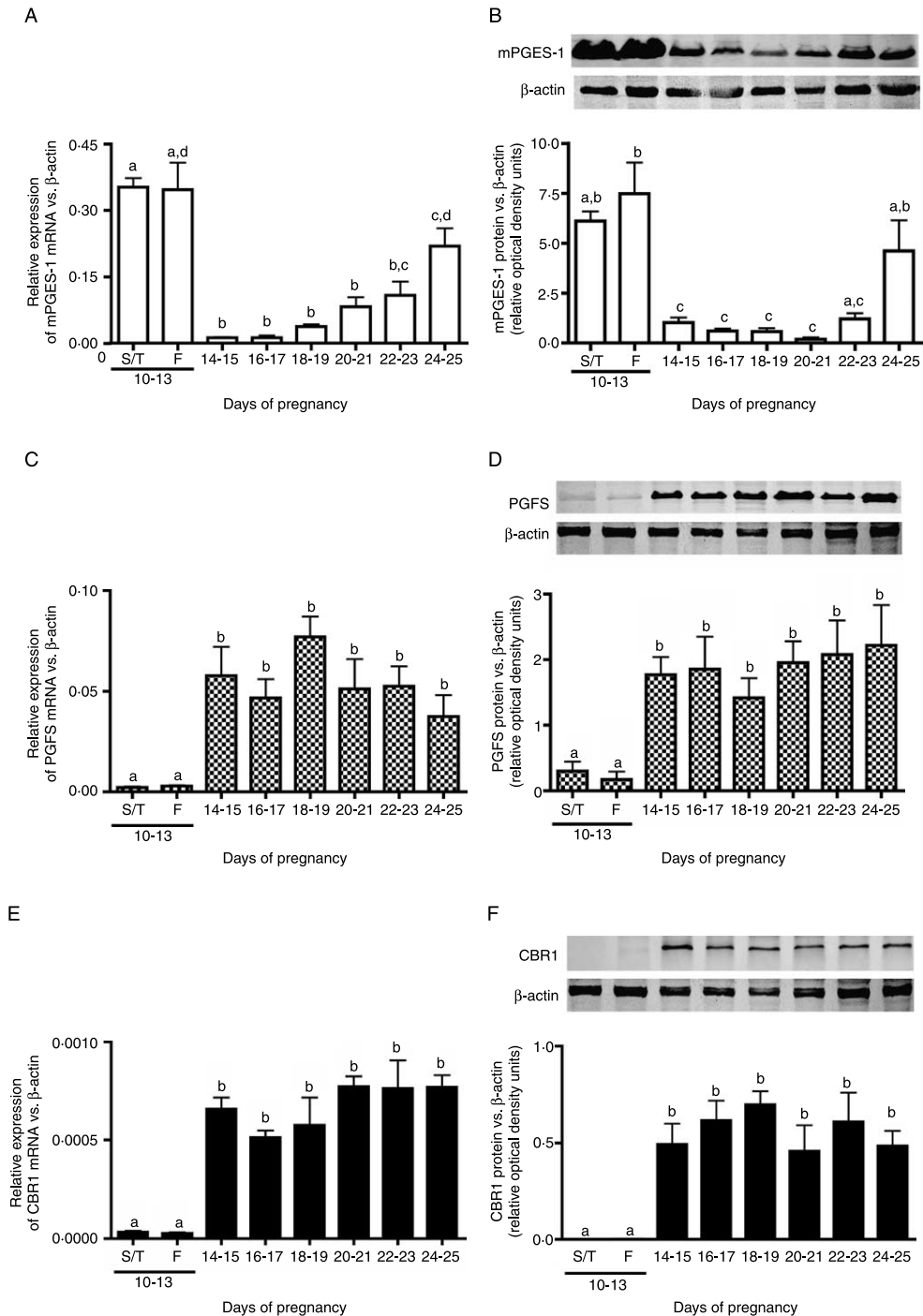


Figure 2 Expression of mPGES-1 (A and B), PGFS (C and D), and CBR1 (E and F) in porcine conceptus/trophoblast during early pregnancy. Quantification of mPGES-1 (A), PGFS (C), and CBR1 mRNA (E) by real-time PCR expressed as the mean \pm s.e.m. ratios relative to β -actin. Western blot analyses of mPGES-1 (B), PGFS (D), and CBR1 proteins (F). β -Actin was used as an internal control for protein loading. Band intensity of mPGES-1, PGFS, and CBR1 was standardized to β -actin band intensity and presented as the mean \pm s.e.m. For each group, conceptuses/trophoblasts collected from three to seven uteri were analyzed. The representative samples of day 10 to day 13 of pregnancy were divided into two groups according to morphology: spherical, tubular (S/T), and filamentous (F). Means with different letters indicate significant differences ($P < 0.05$).

Table 2 Concentration of prostaglandin E₂ (PGE₂), prostaglandin F_{2α} (PGF_{2α}), and PGFM (ng/mg protein) in conceptuses during peri-implantation period

	Days 10–13 S/T ^a	Days 10–13 F ^a	Days 14–15	Days 16–17	Days 18–19	Days 20–21	Days 22–23	Days 24–25
PGE ₂	25.4 ± 6.5 [†]	28.1 ± 9.5 [†]	5.9 ± 1.1 [†]	5.6 ± 1.0 [†]	4.8 ± 0.1 [†]	12.5 ± 2.9 ^{*,†}	11.0 ± 3.9 ^{*,†}	4.3 ± 1.5 [†]
PGF _{2α}	10.8 ± 3.2	16.5 ± 5.6	9.4 ± 3.3	11.1 ± 3.0	6.0 ± 1.5	9.8 ± 3.3	8.7 ± 3.8	5.4 ± 0.8
PGFM	2.1 ± 0.4	3.7 ± 1.0	4.6 ± 1.8	6.2 ± 2.1	4.7 ± 0.9	10.2 ± 4.5	3.4 ± 1.0	1.3 ± 0.3

*[†]Means with different superscripts in a row indicate significant differences in PGE₂ concentration ($P < 0.05$).

^aConceptuses collected from day 10 to day 13 of pregnancy were divided into two groups according to morphology: spherical, tubular (S/T), and filamentous (F).

endometrium, brain, and CL. Low CBR1 protein expression was observed in heart, 20-day embryo, and lung.

Expression of CBR1 in endometrium during the estrous cycle and early pregnancy

Endometrial CBR1 mRNA content was affected by day ($P < 0.001$) but not by reproductive status (Fig. 5A). CBR1 mRNA levels did not differ in endometrium during the estrous cycle (days 1–21) and on days 5–23 of pregnancy. However, increase of the transcript levels occurred on days 24–25 of gestation when compared with all other examined days of the estrous cycle and pregnancy ($P < 0.001$).

A day × reproductive status interaction was detected for endometrial CBR1 protein expression ($P < 0.004$). Endometrial expression of CBR1 protein was affected by day ($P < 0.001$) and by pregnancy status ($P < 0.006$; Fig. 5B). CBR1 protein levels were low from day 1 to day 9 of the estrous cycle. Afterward, CBR1 protein content in endometrium increased and was the highest on days 16–17 of the estrous cycle (when compared with days 1–9 and 18–21 of the estrous cycle, $P < 0.05$; Fig. 5B). During early pregnancy, endometrial expression of CBR1 protein was significantly lower on days 10–11 when compared with days 5–9, 18–19, and 24–25 post-mating (Fig. 5B). CBR1 protein content in endometrium on days 10–11 of pregnancy was also significantly decreased when compared with days 10–11 of the estrous cycle (Fig. 5B). Moreover, the endometrial protein expression of the studied enzyme on days 16–17 of the estrous cycle was significantly higher than on respective days of pregnancy.

Discussion

Achieving an optimal PGE₂/PGF_{2α} ratio is essential for luteolysis or maintenance of the CL, which are the critical events in domestic animal female reproduction. Porcine CBR1 is the enzyme which exhibits PG 9-ketoreductase activity and can modulate both PG concentrations. We found a CBR1 band of ~31.5 kDa to be present in every porcine tissue analyzed but its abundance varied widely. CBR1 was most abundantly expressed in kidney, liver, and oviduct. The results are consistent with previous studies demonstrating that CBR1 is expressed in many tissues in the male pig in the

neonatal stage (Kobayashi *et al.* 1996). Until now, PG 9-ketoreductase activity has also been reported in some female reproductive tissues like ovary, uterus, and placenta in different species (Watson *et al.* 1979, Niesert *et al.* 1986,

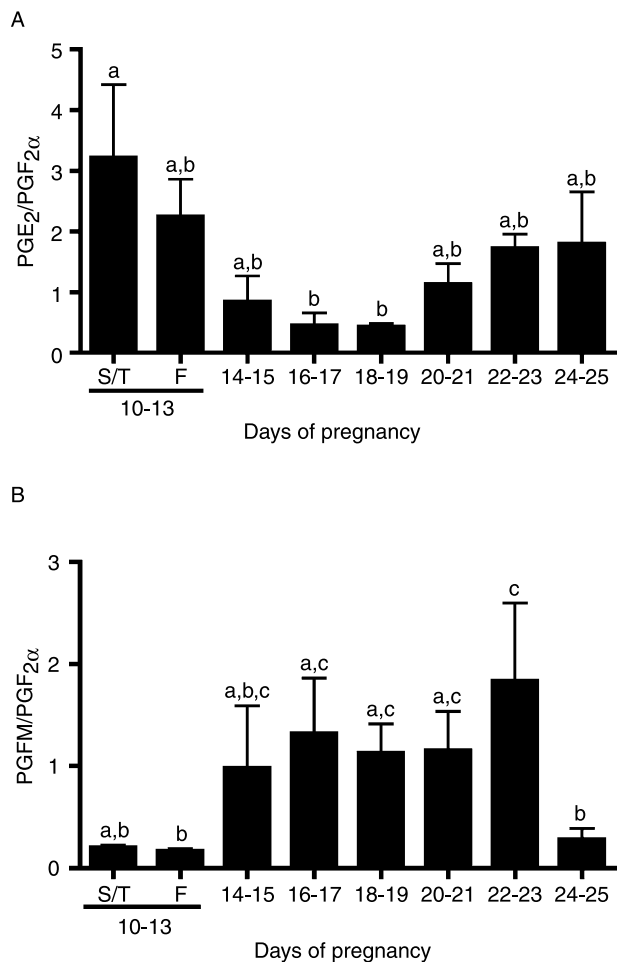


Figure 3 The ratio of PGE₂ to PGF_{2α} (A) and PGFM to PGF_{2α} (B) in porcine conceptus and trophoblast tissues during early pregnancy. Values are expressed as the mean ± S.E.M. For each group, conceptuses/trophoblasts collected from three to seven uteri were analyzed. Conceptuses collected from day 10 to day 13 of pregnancy were divided into two groups according to morphology: spherical, tubular (S/T), and filamentous (F). Means with different letters indicate significant differences ($P < 0.05$).

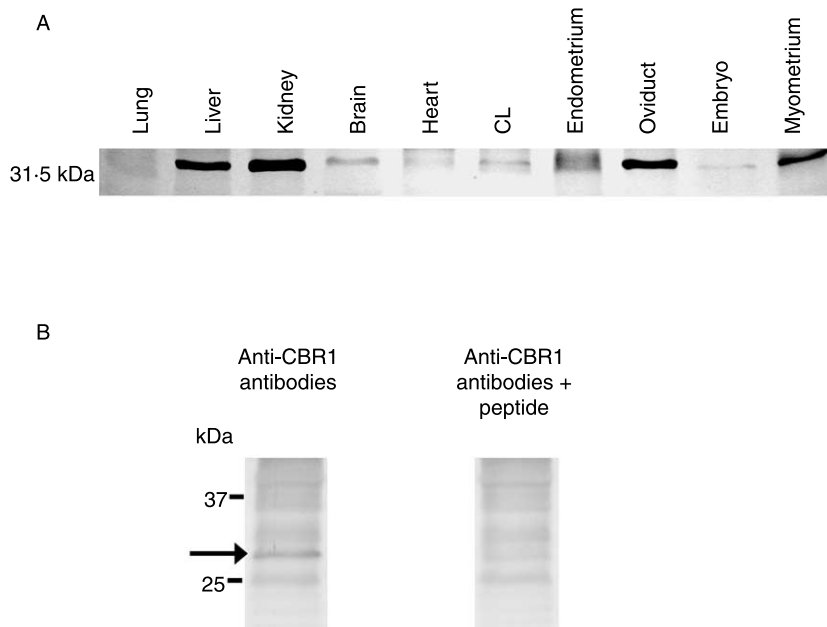


Figure 4 (A) Tissue distribution of porcine CBR1 examined by western blot analysis using polyclonal antibodies against CBR1. Molecular weight of protein is indicated on the left. B, Specificity of the anti-CBR1 antibodies. Proteins (30 µg) prepared from porcine conceptus were analyzed by anti-CBR1 antibodies or anti-CBR1 antibodies blocked by the immunogenic peptide. The antibodies detected a 31.5 kDa protein (B, arrow).

Beaver & Murdoch 1992, Kankofer & Wiercinski 1999). Moreover, the presence of PG 9-ketoreductase mRNA was detected in CL and epithelial cells of bovine endometrium (Asselin & Fortier 2000). The wide distribution of porcine PG 9-ketoreductase (CBR1) may reflect its broad substrate specificity. The studied enzyme reduces keto groups not only on PGs but also on androgens, progestins as well as aldehydes and ketones on a large number of xenobiotics (Tanaka *et al.* 1992, Nakajin *et al.* 1997). Nevertheless, the physiological role of CBR1 is not well determined.

In the present study, for the first time, CBR1 protein and mRNA were identified in the porcine uterine endometrium and conceptus. One potential mechanism by which the PGE₂/PGF_{2α} ratio during the maternal recognition of pregnancy could be changed in favor of the luteoprotective/antiluteolytic PGE₂ may be the direct contribution of the conceptus to PGE₂ synthesis (Davis *et al.* 1983, Wilson *et al.* 2002). Not only CBR1 but also other PG synthesis enzymes were detected in the conceptus indicating that, indeed, porcine blastocyst and trophoblast tissue has potential for PG synthesis. Both PGHS-1 and PGHS-2 mRNA were present in conceptus and trophoblast tissues during the peri-implantation and early placentation stages of gestation. Furthermore, expression of both isoforms was developmentally regulated. PGHS-1 mRNA was significantly lower in spherical/tubular and filamentous conceptuses on days 10–13 of pregnancy. Until now, PGHS-1 protein has been demonstrated in human and mouse pre-implantation embryos (Wang *et al.* 2002, Tan *et al.* 2005).

The present study revealed that PGHS-2 mRNA was significantly elevated in day 10–13 filamentous conceptuses when compared with conceptus/trophoblast tissues collected from day 14 to day 17 and from day 22 to day 25 of pregnancy. Similarly, Wilson *et al.* (2002) demonstrated enhanced PGHS-2 transcript content in day 12 filamentous porcine conceptuses, but in these studies no mRNA of the enzyme was detected in spherical blastocysts from day 11 to day 12 of pregnancy. In contrast, the present report revealed moderate PGHS-2 transcript abundance also in spherical and tubular blastocysts from day 10 to day 13. However, there was a wide variation in mRNA levels among day 10–13 spherical, tubular, and filamentous conceptuses. The difference in these results can be explained due to the much higher sensitivity of the real-time RT-PCR method we applied than the ribonuclease protection assay used in the cited studies. The present study is consistent with reports showing high expression of PGHS-2 protein in ovine and mouse pre-implantation embryos (Charpigny *et al.* 1997, Tan *et al.* 2005).

Our findings provide the first evidence that conceptus PGE synthase is upregulated during trophoblastic elongation and the maternal recognition of pregnancy in any domestic species. The higher mPGES-1 expression in 10- to 13-day conceptuses coincided with elevated PGE₂ content in blastocysts (Davis *et al.* 1983), uterine lumen (Davis & Blair 1993, Ashworth *et al.* 2006), and utero-ovarian circulation in the pig (Christenson *et al.* 1994). It suggests that PGE₂ produced via PGHS-2 and mPGES-1 in conceptus may be involved in CL control. The present study also correlates with

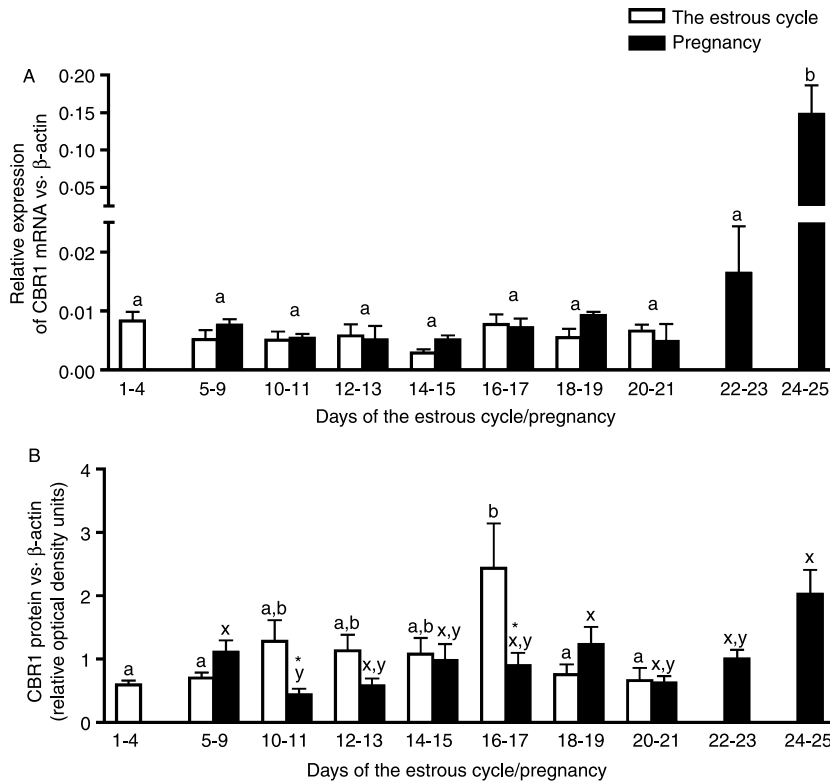


Figure 5 Comparison of CBR1 expression in endometrium between cyclic (white bar) and pregnant gilts (black bar). (A) Quantification of CBR1 mRNA by real-time PCR, expressed as the mean \pm s.e.m. of ratios relative to β -actin. B, Western blot analyses and densitometry of CBR1 protein. β -Actin was used as an internal control for protein loading. Band intensity of CBR1 was standardized to β -actin band intensity and presented as the mean \pm s.e.m. For each group, three to nine uteri were analyzed. Days with different superscripts for CBR1 mRNA abundance are statistically different ($P < 0.001$). Endometrial expression of CBR1 protein was affected by day \times pregnancy status ($P < 0.004$). Values for abundance of endometrial CBR1 protein with different letters differ within the estrous cycle ($a < b$; $P < 0.05$) or early pregnancy ($y < x$; $P < 0.05$). The asterisk (*) represents differences ($P < 0.05$) between cyclic and pregnant gilts in respective days of the estrous cycle and pregnancy.

the report showing that PGE₂ treatment at the blastocyst stage could accelerate and enhance spreading of the trophoblast *in vitro* (Chan 1991). Our findings revealed two periods of increased conceptus/trophoblast mPGES-1 expression (days 10–13 and 22–25), which correspond with profile of the PGE₂/PGF_{2 α} ratio in peri- and post-implantation conceptuses. A similar pattern of mPGES-1 expression has recently been described in endometrium also during early pregnancy in the pig (Waclawik *et al.* 2006). However, the changes of PGE synthase content in endometrium are less dynamic than in conceptus.

PGE synthase expression pattern in conceptus also correlates with biphasic profiles of estrogen synthesis and secretion by blastocysts (Geisert & Yelich 1997). Even spherical 5–7 mm blastocysts from day 10 to day 11 after fertilization are capable of enhanced synthesis of estradiol-17 β and other estrogens (Fisher *et al.* 1985, Pusateri *et al.* 1990). This suggests that mPGES-1 in both endometrium

and conceptus may be stimulated by conceptus estrogens. Moreover, it was demonstrated that estrogens may be an important factor in increase of PGE₂ synthesis (Geisert *et al.* 1982b) and the PGE₂/PGF_{2 α} ratio in porcine endometrium (Ziecik 2002).

In contrast to PGE synthase, both enzymes involved in PGF_{2 α} production were expressed in conceptus at very low level during the maternal recognition of pregnancy. Among pre-implantation day 10–13 conceptuses, no differences were found among spherical, tubular, and filamentous forms in expression of PGFS and PG 9-ketoreductase (CBR1). Interestingly, the PGE₂/PGF_{2 α} ratio in peri-implantation conceptuses may be reverse index of PG 9-ketoreductase activity and also reflects activities of mPGES-1 and PGFS in conceptus. Therefore, profile of the PGE₂/PGF_{2 α} ratios did not correspond exactly to inverse expression pattern of CBR1 in conceptus.

Upregulation of PGHS-2 and mPGES-1, and down-regulation of PGFS and CBR1 expression in day 10–13

conceptuses may play an important role in increase of the PGE₂/PGF_{2α} ratio, which is a pivotal event in the maternal recognition of pregnancy in the pig. The present results are consistent with the reports, suggesting that conceptus secretes much more PGE₂ than PGF_{2α} during this period (Davis *et al.* 1983, Wilson *et al.* 2002). Moreover, relatively stable levels of mPGES-1, PGFS, and CBR1 in CL (A Waclawik, unpublished) and moderate changes of PGF and PGE synthases in endometrium (Waclawik *et al.* 2006) indicate a significant contribution of pre-implantation conceptus to synthesis of PGE₂ during the maternal recognition of pregnancy in the pig.

Interestingly, after initiation of implantation, expression of PGFS and CBR1 in conceptus and trophoblastic tissues increased sharply and remained high by days 24–25 of pregnancy, which correlates with the high PG concentrations in uterine lumen in implantation period (Davis & Blair 1993, Ashworth *et al.* 2006). These findings also correspond with reduced luteal PGF_{2α} receptors concentration on day 14 in pregnant when compared with cycling pigs, which may lead to decreased luteal sensitivity of pregnant CL to PGF_{2α} (Gadsby *et al.* 1993). Furthermore, changes of the PGFM/PGF_{2α} ratios in the peri-implantation conceptuses indicate that 15-hydroxyprostaglandin dehydrogenase activity is low in 10- to 13-day conceptuses and increased during implantation and post-implantation period (days 16–23 of pregnancy). Higher levels of 15-hydroxyprostaglandin dehydrogenase activity after day 14 of pregnancy suggest enhanced metabolism of PGF_{2α} during implantation, which may decrease luteolytic effect of PGF_{2α} and, in such a way, maximize the biological effect of luteotropic PGE₂. Changes of PGFM/PGF_{2α} ratio correspond with CBR1 expression profile in conceptus and report that CBR1 can also exhibit some 15-hydroxyprostaglandin dehydrogenase activity (Chang & Tai 1981).

The transient elevated expression of mPGES-1 that we found in conceptus/trophoblast is in agreement with studies on the significant role of mPGES-1 in implantation in other species (Ni *et al.* 2002, Wang *et al.* 2004). It is likely that PGE₂ produced in the conceptus could exert an immunomodulatory effect (Parhar *et al.* 1989) and act in paracrine manner via endometrial PGE₂ receptors (Kennedy *et al.* 1986), resulting in the local increase of endometrial vascular permeability and preparation for angiogenesis and placentation (Hamilton & Kennedy 1994, Yang *et al.* 1997).

Moreover, profiles of mPGES-1 and PGFS expression in conceptus/trophoblast are highly correlated with changes of expression of both synthases in endometrium recently reported by us (Waclawik *et al.* 2006). The similar patterns of PG synthase expression may be a result of interactions between conceptus and endometrium, which are essential for maternal recognition of pregnancy, implantation, and placentation.

In the present study, CBR1 mRNA and protein were identified not only in the conceptus but also in the uterine endometrium. Interestingly, it was shown more than 20 years

ago that PGE₂ intrauterine infusion increases PGF_{2α} concentration immediately in utero-ovarian vein in the cyclic gilt (Okrasa *et al.* 1985). We believe that it can now be explained by activity of endometrial CBR1, the enzyme that converts PGE₂ into PGF_{2α} when the concentration of estrogens in the circulation is low. Furthermore, in the present study, a significant decrease of CBR1 protein expression was observed in the endometrium on days 10–11 of pregnancy when compared with the corresponding days of the estrous cycle. It is consistent with the findings demonstrating inhibition of activity of PG 9-ketoreductase in sheep endometrium during the maternal recognition of pregnancy (Beaver & Murdoch 1992). Therefore, the present report supports the hypothesis that CBR1 expression changes in the endometrium are involved in the increase of the PGE₂/PGF_{2α} ratio in the uterus during maternal recognition of pregnancy in the pig.

The data presented in this report demonstrated that CBR1 was upregulated in the endometrium during the end of luteolysis. Comparison of endometrial CBR1 protein levels between day 16–17 cyclic and pregnant gilts indicated significantly higher expression of this enzyme in the cyclic animals. Moreover, it has previously been shown that PGFS is expressed abundantly on days 13–15 of the estrous cycle (Waclawik *et al.* 2006). It appears that CBR1 is not involved in initiation of luteolysis like PGFS but may rather play a role in successful completion of luteal regression.

The results of present study provide the first direct evidence to support the hypothesis that reciprocal expression of PGHS-2, mPGES-1, and the downstream enzymes involved in PGF_{2α} production in 10- to 13-day conceptuses could be important in increase of the PGE₂/PGF_{2α} ratio during the maternal recognition of pregnancy in the pig. Our findings indicate that another potential mechanism of luteolysis inhibition in the pig may be downregulation of endometrial CBR1 protein expression. This study suggests possible conceptus–endometrium interaction in the increase of the PGE₂/PGF_{2α} ratio in uterine lumen at this critical period. Moreover, high conceptus expression of PGFS, mPGES-1, and CBR1 after initiation of blastocyst attachment may indicate involvement of these enzymes in implantation and early placentation in the pig.

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