Developmental regulation of prostacyclin synthase and prostacyclin receptors in the ovine uterus and conceptus during the peri-implantation period

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Abstract

This study documents the expression of prostacyclin (PGI2) synthase (PTGIS) and PGI2 receptors in the trophoblast and uterus of the ewe at the time of maternal recognition of pregnancy (i.e. days 7, 9, 12, 14 and 17). The membrane receptor for PGI2 (PTGIR) and the nuclear receptors, i.e. peroxisome proliferator-activated receptors (PPAR) and their heterodimer partners the retinoid X receptors (RXR), were analysed. In the endometrium, PTGIS transcript and protein were expressed at day 9 of pregnancy and levels declined from days 12 to 17. Immunohistochemistry and in situ hybridization indicated that PTGIS was mainly located in the luminal epithelium of the endometrium. Endometrial PTGIR, PPARA, PPARG and RXRG expression was regulated during the peri-implantation period whereas PPARD, RXRA and RXRB were consistently expressed. In the trophoblast, PTGIS transcript levels rose as development progressed and peaked at day 17. PTGIR and PPARA transcripts peaked before day 12 and then declined and became nearly undetectable by day 17, whereas PPARD and PPARG transcript levels rose steadily from days 12 to 17. Because the PPARs and the RXRs display different expression profiles, we suggest that different heterodimers may form and support distinct functions as development proceeds. Our results also underline the importance of PTGIS and PPARD in the trophoblast and PTGIR in the uterus, suggesting that PGI2 is of both uterine and trophoblastic origin and is involved in a complex signalling pathway at around the time of implantation in the ewe.


Introduction

The significance of prostaglandin during embryo implantation has been demonstrated in cyclooxygenase-2 (PTGS2)-deficient mice (Lim et al. 1997) which exhibit implantation defects. The cyclooxygenases convert arachidonic acid into prostaglandin (PG) H2 which is the substrate for the specific isomerases that generate biologically active prostaglandins (Mitchell 1992). PTGS2 has been identified in the uterus and embryo of various species. In ruminants, PTGS2 is strongly expressed anddevelopmentally regulated in both trophoblast cells of the conceptus and the endometrium, from the time of hatching to implantation (Charpigny et al. 1997a,b, Emond et al. 2004). Studies in the mouse have focused on the prominent role of uterine PTGS2-derived prostaglandins at implantation because PTGS2 is present in the uterine luminal epithelium and subepithelial stroma around the implanting blastocyst (Chakraborty et al. 1996). However in the ewe, prostaglandins are produced by the uterus and trophoblast (Charpigny et al. 1997b, 1999).

In mice, prostacyclin (PGI2) is the prominent prostaglandin synthesized at uterine implantation sites (Lim et al. 1999). PGI2 is critical to endometrial decidualization and embryo implantation, because a PGI2 analogue injected into PTGS2-deficient mice restored implantation defects (Lim et al. 1999). In ruminants, both the endometrium and the trophoblast produce PGI2 (Charpigny et al. 1997b, 1999). In ruminants, endometrial PGE2 and PGF2α are respectively well known for their luteotrophic and luteolytic activities (Goff 2004) whereas PGI2 has been the subject of little investigation. Prostaglandins act mainly via G-protein-coupled membrane receptors (Narumiya et al. 1997, Breyer et al. 2001), including the PGI2 receptor PTGIR (Katsuyama et al. 1994). Recent evidence has shown that prostaglandins can also act via nuclear receptors (Forman et al. 1997); this is consistent with the perinuclear localization of PTGS2 (Morita et al. 1995, Van der Weiden et al. 1996, Marvin et al. 2000a), which has been demonstrated in many models as well as in the ovine trophoblast (Charpigny et al. 1997b).
PPARs (peroxisome proliferator-activated receptors) are members of the nuclear hormone receptor superfamily that act as ligand-inducible transcription factors by interacting with the PPRE (peroxisome proliferator response element) on the promoter of target genes. Three isotypes are known: PPARα, PPARδ and PPARγ being encoded by different genes (Desvergnes & Wahl 1999). PPARα was recently identified in the human full-term placenta (Wang et al. 2002). However, the knock-out of PPARα in mice produces no reproductive defects (Ding et al. 2003). PPARδ displays marked induction in the mouse uterus at the time of implantation (Lim et al. 1999). The knock-out of PPARα in mice produces placental defects and mid-gestation lethality but no implantation failures (Ding et al. 2003). The PPARγ transcript is expressed in human placential tissues (Marvin et al. 2000b) and in bovine embryos (Mohan et al. 2002). Knock-out experiments in mice have shown that PPARγ regulates terminal differentiation of the trophoblast and that PPARγ-dependent trophoblast functions are involved in placental vascularization (Barak et al. 1999).

PPARs heterodimerize with retinoid X receptors (RXRs) to regulate target gene activation. The heterodimerization of PPARs with RXRs is a prerequisite for their DNA binding activity and target gene activation. Abundant expression of RXRA transcript is reported in mouse decidua (Mangelsdorf et al. 1990) and uterus (Lim et al. 1999) during peri-implantation, and in the cytotrophoblast during human implantation (Tarrade et al. 2000). RXR-null mutant mice are embryonic lethal (Kastner et al. 1994). RXRA and RXRB transcripts are both expressed in the bovine embryo during early development (Mohan et al. 2002).

The first step in understanding the role of PGII signaling at the time of the maternal recognition of pregnancy and the initiation of implantation in ruminants is to assess the expression of the different partners involved in PGII synthesis and signal transduction. The present study establishes the expression of PGII synthase (PTGIS) and receptors (PTGIR, PPARs and RXRs) in the ovine uterus and conceptus during the peri-implantation period. Our results demonstrate that the trophoblast is the predominant tissue in which PTGIS is expressed and they suggest a temporal sequential role of PGII receptors during the early stages of development and implantation.

Materials and Methods
Sample collection
All procedures relative to the care and use of animals were approved by the French Ministry of Agriculture in line with French regulations (decreet dated 19 April 1988) concerning animal experimentation.

Ewes of the Préalpes-du-Sud breed were used. Oestrus was synchronized using intravaginal sponges containing 60 mg 6α-methyl-17α-acetoxypregesterone (Intervet, Angers, France) for 14 days, as previously described (Peterson et al. 1976). On the day of sponge withdrawal, the ewes received one injection of 500 IU equine chorio- nic gonadotrophin (eCG; Intervet). Oestrus was observed 48 h later (day 0), and the ewes were mated twice. On days 7 (n = 4), 9 (n = 7), 12 (n = 6), 14 (n = 7) and 17 (n = 3) of pregnancy, the ewes were slaughtered. The uterus was removed and the concepti were collected by flushing the uterine horns with PBS at 37°C. The development of concepti was confirmed by binocular examination, the embryonic area was discarded and trophoblast cells washed in cool PBS. Luminal uterine epithelial cells corresponding to the superficial endometrium were collected by scraping, as described previously (Charpigny et al. 1999).

Uterine cells and trophoblasts were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. Pieces of the uterus horn and conceptus were preserved for immunohistochemistry and in situ hybridization (ISH). The tissues were fixed by 4% (w/v) paraformaldehyde for 2–3 h at 4°C, washed in cool 0.1 M phosphate buffer pH 7.5 and treated in serial baths of 15% (w/v) sucrose and 18% (w/v) sucrose in phosphate buffer. Tissue pieces were embedded in Tissue Tek, frozen in liquid nitrogen vapour and stored at −80°C.

RNA purification and quantification
Total RNA was extracted using a phenol-based method derived from that described by Chomczynski and Sacchi (1987), and then quantified by spectrophotometry. RNA integrity was verified by non-denaturing agarose electrophoresis, except for day-7, -9 and -12 embryos. The quantification of low levels of RNA in day-7 to day-12 embryos was performed as previously described, but with some modifications (Harley 1987). Two different dilutions (6× SSC, 7.4% (v/v) formaldehyde) of each sample were immobilized on a nylon filter along with serial dilutions of an RNA scale using a slot blot device and fixed with 0.4 M NaOH. OligoDT (Invitrogen) was radio-labelled with [γ-32P]dATP using a T4 polynucleotide kinase (Invitrogen) and 2.5 × 106 c.p.m. was hybridized onto the filters (25°C for 5 h in 5× SSC, 5× Denhardt’s solution, 0.01 M Na2HPO4, 1 mM Na3H2PO4). After washing (25°C, 2× SSC) the filters were exposed to a phosphor imaging screen, visualized after scanning with a phosphor imager (FLA3000; Fujifilm, Courbevoie, France) and analysed by Advanced Image Data Analysis software (Raytest, Courbevoie, France).

Semi-quantitative RT-PCR
Three pools of seven day-7 embryos, three pools of five day-9 embryos, six individual day-12 trophoblasts, seven individual day-14 trophoblasts and three individual day-17 trophoblasts were studied. Four day-7, four day-9, four day-12, four day-14 and three day-17 endometrial
samples were analysed. Reverse transcription was run on total RNA at 37 °C with an oligo(dT) primer and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen). One microgram of superficial endometrial total RNA was used; 250 ng of trophoblastic total RNA corresponding to seven day-7 and five day-9 embryos were introduced into the reaction in order to process all the embryos with the same amount of RNA. PCR was performed using 5% of RT products. Reaction mixtures for PCR, including Taq polymerase (QBioGen, Illkirch, France) were prepared as suggested by the manufacturer. The PCR conditions were as follows: 1 min denaturing at 94 °C, and then the appropriate number of cycles of denaturing at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s. The primers used are shown in Table 1. The primers were designed using bovine or human sequences when the ovine sequence was not available. Three numbers of PCR cycles were performed for each reaction in order to demonstrate the linearity of amplification and to enable semi-quantitative comparisons. At the appropriate cycle, 5 μl of the PCR products were subjected to electrophoresis on a 2% (w/v) agarose gel. The identity of all PCR products was confirmed by DNA sequencing (Genome Express, Montreuil, France) and sequence homology analysis using the Basic Local Alignment Search Tool (Altschul et al. 1997). PCR products were transferred onto Hybond N + nylon filters along with the purified probe as a size control (Amersham) in 0.4 M NaOH. [α-32P]dCTP-labelled probes were generated from purified PCR products (ReadyPrim II, Amersham), then purified (GenClean turbo, QBioGen) and hybridized overnight at 65 °C in 0.5 M phosphate buffer, 7% (w/v) SDS onto the filters. They were then washed with 0.1 X SSC, 0.1% (w/v) SDS and exposed for 2 h against a phosphor imaging screen. Filters were scanned with a FLA3000 (Fujifilm) phosphor imager and the signal analysed by Advanced Image Data Analysis (Raytest) software. Results were normalized with β-actin (ACTB), which exhibits no statistical difference during the course of development, and expressed as a percentage of the maximum mean value.

Western blot analysis of PTGIS

Frozen uterine and trophoblastic tissues were homogenized in a 50 mM Tris–HCl pH 7.4 lysis buffer containing 5 mM EDTA, 16 mM CHAPS (3-((3-cholamidopropyl)-dimethylammonioli)-1-propane-sulphonate, 1 mM benzamidine-HCl, 1 mM phenylethylsulphonyl and soybean trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), apro tin (10 μg/ml) (Sigma). After sonication and centrifugation at 15 000 r.p.m. for 10 min at 4 °C, the protein concentration of cellular extracts was determined using the BioRad protein assay. Total proteins (5 μg for trophoderm, 30 μg for uterine cells) were electrophoresed in 12% SDS-PAGE, and electro-transferred onto a Hybond-P, PVDF membrane (Amersham). Membranes were submitted to immunodetection using rabbit polyclonal antibody (Cayman Chemical, Product number 160640; Spi-Bio, Massy, France) raised against a synthetic peptide from the bovine PTGIS sequence. The antibody was diluted 1:2500 in 20 mM Tris–HCl, 500 mM NaCl, 5% (w/v) low-fat milk and 0.1% (w/v) Tween 20. Peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Interchim, Montluçon, France) was diluted at 1:10000 for immunodetection. The immunoreactive signals revealed by the ECL + detection kit (Amersham), were visualized using a FUJIFILM LAS-1000 Camera System (Fujifilm) and analysed by Advanced Image Data Analysis software (Raytest). Five to ten micrograms of endothelium extract containing PTGIS were used as a standard in order to normalize and compare the amounts of PTGIS. The molecular mass of the identified protein was calculated

Table 1 PCR conditions and sequence references for primers.  

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using the Provieve color protein markers (BMA product, Tebu-bio, Le Perray en Yvelines, France).

**ISH of PTGIS**

PCR products were cloned into a pCR4TOPO vector (TOPO TA Cloning Kit, Invitrogen) according to the manufacturer’s instructions. The plasmid vector included T3 and T7 RNA polymerase promoters. The sequence of construction was verified and the insertion direction determined. Digoxigenin (DIG)-labelled UTP cRNA probes were generated by in vitro transcription from 200 ng of the PCR template using T7 polymerase for antisense and T3 polymerase for sense, according to the manufacturer’s protocols (Promega). Probe concentrations were evaluated with DIG-labelled control RNA (Roche). The ISH procedure was performed as described elsewhere (Yang et al. 1999). Frozen sections (10 μm thick) from conceptus and uterine tissues were stored under vacuum in desiccate at 4°C for no longer than 4 weeks. Slides were delipidized for 5 min with chloroform and then re-hydrated in PB. Major pre-treatments consisted of permeabilization with 10 μg/ml proteinase K and acetylation with 0.25% (v/v) acetic anhydride in triethanolamine 0.1 M, pH 8. Sections were hybridized overnight at 65°C with antisense or sense probes (70–100 μl of 200 ng/ml) in hybridization buffer (2 × SSC, 2.5% (w/v) dextran sulphate, 5 × Denhardt’s solution, 4 mM EDTA, 50% (v/v) deionized formamide, 50 μg/ml yeast tRNA and 0.25 mg/ml hydrolysed salmon sperm DNA). Hybrids formed in situ were revealed using a DIG-RNA detection kit (Roche) by incubation for 2 h with a sheep anti-DIG-alkaline phosphatase-conjugated antibody diluted at 1:2500 and revealed overnight with NBT/BCIP as the substrate. Photomicroscopy was performed as described previously for ISH.

**Immunohistochemistry**

Immunohistochemistry was performed with the Vectastain Elite ABC peroxidase system (Vector Laboratories, Biovalley, Marne la Vallée, France). Air-dried frozen tissue sections were rehydrated with 0.1 M phosphate buffer (PB) containing 0.2% (w/v) BSA. The slides were heated for 10 min in 10 mM citrate pH 6 at 85°C, cooled to ambient temperature, washed in PB and then treated for 30 min in 0.1% (v/v) hydrogen peroxide in H2O to quench endogenous peroxydase activity. Rabbit polyclonal antibodies to PTGIS (Cayman Chemical product number 160640), PPARD (ABR product number PA1-823; Ozyme, Saint Quentin Yvelines, France) and PPARG (ABR product number 2492) and goat polyclonal antibodies to PPARA (Santa Cruz product number SC-1982; Tebu Bio, Le Perray en Yvelines, France) were diluted 1:5000 (PTGIS), 1:3500 (PPARD), 1:500 (PPARG) and 1:100 (PPARA) in PB containing 2% (w/v) BSA and donkey serum diluted 1:100. Sections were incubated overnight in a cold damp box. After two washes, the biotinylated secondary antibody raised in donkey serum (Jackson Immunoresearch, Interchim, Montluçon, France) diluted 1:1000 in PB containing 2% (w/v) BSA was incubated for 1 h. An avidin–biotin peroxidase complex in 50 mM Tris–HCl pH 7.6 was applied for 1 h. Staining was revealed after 5 min in a bath of diaminobenzidine tetrachloride (DAB) 0.5 mg/ml in 50 mM Tris–HCl pH 7.6, 0.15 M NaCl with 0.5% (w/v) ammonium nickel(II) sulphate and 0.03% (v/v) H2O2. The sections were post-fixed with cooled 2% paraformaldehyde (w/v) in PBS, rinsed with bidistilled water, and mounted with Aquaperm Mounting medium (CML, Nemours, France) and Entellan (Le Puy, France). The colorimetric hybridization signal were transformed into green levels (0–255) and the relative optical density was quantified using image analysis software (Olympus-DP50 microscope, digital camera system and software (Olympus SA, Rungis, France). The colorimetric hybridization signal were transformed into green levels (0–255) and the relative optical density was quantified using image analysis software (ImageTool-UTHSCSA; Microsoft Corporation). Signal intensity was the average density over a calibrated area. It was compared with background values given by hybridization with sense probes. Analysis was performed for at least two sections and six areas per animal. Three to five animals were included in the study.

**Statistical analysis**

Results were analysed using one-way ANOVA (Systat software, Erkrath, Germany) followed by a post-hoc Bonferroni pair-wise comparison. When appropriate, data were subjected to linear regression where the day of pregnancy was the independent variable. Statistical significance was accepted at P < 0.05. The data are presented as means ± S.E.M.

**Results**

**Characterization of nucleic acid probes**

Primer information for PTGIS, PTGIR, PPARA, PPARG, RXRA, RXRB, RXRG is summarized in Table 1. The use of these primers on ovine tissues allowed for amplification of products of the predicted size (Fig. 1). The isolated ovine cDNA sequences exhibited 85% (PTGIS), 97% (PPARD), 94% (PPARG) and 90% (RXRG) homology with human sequences and 96% (PTGIS), 94% (PPARA), 91% (RXRA) and 92% (RXRB) homology with bovine cDNA sequences. These ovine cDNAs were used as probes in the hybridization procedures.

**Expression profile of PTGIS in the uterus and trophoblast**

Patterns of PTGIS transcript and protein expression levels during early pregnancy were analysed by
semi-quantitative RT-PCR and Western blot on trophoblast and endometrial samples.

Figure 2 shows a Western blot analysis for the PTGIS protein in ovine tissues. No signal was detected when only the second antibody was used (Fig. 2A), showing absence of unspecific signal. In contrast, an expected single signal was detected at 56 kDa with the anti-PTGIS (Fig. 2B).

In the endometrium, the transcript (Fig. 3A) and protein (Fig. 3B) displayed comparable profiles. PTGIS was strongly detected at days 7 and 9, but less expressed on days 14 and 17 – when it was nearly undetectable (P<0.05). At day 12 a divergence was noted between the transcript (which was undetectable) and the protein (which was still expressed).

In the trophoblast, levels of the PTGIS transcript rose (linear regression, P<0.05) from days 7 to 17. Western blot analysis of PTGIS was performed on individual trophoblasts from days 12 to 17. Since the PTGIS signal
could not be detected in less than 1 µg protein, embryos earlier than day 12 were not introduced into the study. PTGIS protein was expressed in trophoblasts from days 12 to 17, but without any significant increase. During the implantation period, the cell content of PTGIS per microgram of total tissue protein was ten times higher in trophoblast cells than in endometrial cells.

**PTGIS distribution in the uterus and trophoblast**

PTGIS mRNA and protein localization in the uterus (Fig. 4) and the conceptus (Fig. 5) were analysed by ISH and immunohistochemistry. The PTGIS transcript and protein were detected in the uterus at high levels in the luminal epithelium, moderate levels in the glandular epithelium and low levels in the stroma. Signals were also detected in the myometrium as well as in the endothelium, as previously described (DeWitt & Smith 1983). The intense luminal epithelium signal at days 7 and 9 gradually declined until day 17, which was consistent with the results obtained using PCR and Western blot. The reductions in transcript and protein expression only applied to the luminal epithelium.

In concepti, the transcript (data not shown) and protein were detected in trophoblast and endoderm cells irrespective of the developmental stage (Fig. 5). However, at a higher magnification, it could be seen that the perinuclear localization of PTGIS protein observed at day 12 evolved towards a more cytoplasmic and apical distribution at days 14 and 17 (data not shown).

**PTGIR expression profile in the uterus and trophoblast**

In the endometrium, the PTGIR transcript was expressed at similar levels from days 7 to 14. A significant, 9-fold induction occurred at day 17 ($P < 0.05$) (Fig. 3C). In the trophoblast, the transcript could not be evidenced using 250 ng total RNA. When 1 µg was added to the RT reaction, the transcript was detected at day 12 but then gradually declined to day 14, becoming almost undetectable at day 17 (linear regression, $P < 0.05$).

**PPAR expression profiles in the uterus and trophoblast**

In the endometrium, PPAR transcript expression followed a linear decline from days 7 to 17 (linear regression, $P < 0.05$) (Fig. 6). The PPARD transcript was detected at all developmental stages with no difference in expression. The PPARG transcript was erratically regulated ($P < 0.05$). Its expression peaked at day 9, decreased afterwards and became strongly detectable again at day 17.

In the trophoblast, all PPAR transcripts displayed differential kinetic profiles ($P < 0.05$). The PPARA transcript was strongly detected at day 7 and then the signal became nearly undetectable between days 9 and 17. The PPARD transcript displayed an opposite expression profile. It was detected at all stages of embryonic development with a regular increase from days 7 to 14 (linear regression, $P < 0.05$). The marked individual variability observed at day 17 suggested that this developmental stage might constitute a pivotal point in PPARD expression. PPARA transcript expression exhibited a gradual increase (linear regression, $P < 0.05$) as development progressed.

**PPAR distribution in the uterus and trophoblast**

PPAR protein localization in the uterus and conceptus was analysed at 14 days of pregnancy by immunohistochemistry (Fig. 7). In the uterus, PPARA was poorly detected. Both PPARD and PPARG proteins were detected in the nucleus of the cells. PPARD protein was mainly expressed in epithelial luminal and superficial glandular cells. A faint signal was also identified in the other uterine compartments. PPARG protein was detected at a strong level in all epithelial compartments and also in the stroma.

In the conceptus, PPARA was detected in very few trophoblastic cells which appear as mononucleate cells. PPARD and PPARG were detected in the nucleus of nearly all trophoblastic cells and in the nucleus of the few endoderm cells. The signal for PPARD was stronger in the trophoblast than in the luminal epithelium.

**RXR expression profiles in the uterus and trophoblast**

In the endometrium, the RXRA transcript was difficult to detect beyond day 9 and displayed no significant differences in expression (Fig. 8). The RXRB transcript was expressed at all developmental stages, with no significant differences. The RXRG transcript displayed a differential ($P < 0.05$) pattern of expression during early development. It was more strongly expressed at day 9 than at all the other stages. In the trophoblast, the RXRA and RXRG transcripts exhibited no significant differences in their expression (Fig. 8). RXRB was detected at all stages and displayed a lower level of expression at days 12 and 14 ($P < 0.05$).

**Discussion**

The present study investigated the expression profiles of the enzyme and receptors of the PGI2 signalling pathway in the ovine uterus and conceptus during the peri-implantation period, which has never been reported in ruminant species. In the endometrium, PTGIS was strongly expressed before day 12 and was down-regulated thereafter. The trophoblast displayed the opposite profile. PTGIS was nearly undetectable before day 12 and was up-regulated thereafter. At the time of implantation, which starts at day 14 of pregnancy in the ewe, PTGIS protein was ten times more abundant in the trophoblast than in the endometrium.

The factors known to control PTGIS expression in the uterus appear to differ as a function of species, tissue and physiological state. In humans, PTGIS transcript levels rise following steroid withdrawal at menstruation (Battersby et al. 1980).
et al. 2004), whereas oestradiol stimulates PGI2 secretion in endometrial stromal cells (Levin et al. 1992) and myometrial cells (Korita et al. 2004). In the sheep, Wu et al. (1999) reported that oestradiol and progesterone treatment had no effect on endometrial and myometrial PTGIS protein expression in ovariectomized animals. However, oestradiol treatment increases PTGIS protein levels in the uterine artery endothelium and in vascular smooth muscle

<table>
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**Figure 4** Localization of PTGIS transcript and protein on representative transversal sections of uteri from days 7 to 17 of pregnancy. My, myometrium; GE, glandular epithelium; LE, luminal epithelium; St, stroma; scale bar, 50 μm. (A) ISH with a DIG-labelled antisense cRNA probe was revealed with an alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP substrate. Negative controls at day 9 were performed using the sense probe. (B) Immunohistochemistry with polyclonal anti-PTGIS antibody was revealed with a peroxidase Elite-ABC Vectastain kit and DAB substrate. The controls at day 9 were performed without the first antibody. Slides were not counter-stained.
A similar regulation in the endometrium is unlikely because we found that the decrease in PTGIS expression coincided with the known rise in oestriol levels associated with the second wave of follicular growth that occurs at day 12 (Cox et al. 1973). The decline in PTGIS transcript levels after day 9 in the endometrial luminal epithelium also coincides with a loss of progesterone receptors in that cell type (Spencer et al. 2004). PTGIS expression and prostacyclin production is induced by tumour necrosis factor (TNF) and interleukin 6 (IL6) (Helliwell et al. 2004). These cytokines are present in the gravid ovine endometrium (Rahman et al. 2004) where they may also contribute to regulating PTGIS. We found that as the trophoblast elongated, from days 12 to 14, PTGIS was developmentally up-regulated. Here again, local mediators may play a role in this regulation as cytokine involvement has been postulated (Hansen et al. 1999). It should be noted that the simultaneous increase in both trophoblast size and PTGIS concentration per cell resulted in an increased capacity for PGI2 synthesis as development proceeds.

There is a correlation between PTGIS expression (this study) and the synthesis of PGI2 in the ovine endometrium and conceptus (Charpigny et al. 1997b, 1999). Thus PGI2 is constantly present in the environment of the conceptus, being first of endometrial origin (days 7 to 9) and then of trophoblastic origin (day 12 and beyond). In the endometrium, PTGIS is co-expressed with PTGS1 (Charpigny et al. 1997a); in the trophoblast, PTGIS is co-expressed with PTGS2 (Charpigny et al. 1997b). We hypothesize that this dual functional coupling between PTGIS and PTGS enzymes may be related to the differences that we found in PTGIS localization: PTGIS was preferentially seen in the peri-nuclear region of the trophoblast and displayed a cytosolic localization in the endometrium. A dual localization for PTGIS had been previously reported (Liou et al. 2000, Ueno et al. 2001). This supports the general observation that PGI2 and other prostanoids produced in the endoplasmic reticulum are secreted towards the extracellular milieu to act as autacoids, whereas the enzymes located on the nuclear envelope produce prostaglandins.

Figure 5 Immunohistochemical localization of PTGIS protein on representative sections of concepti from days 9 to 17 of development. T, trophoblast; En, endoderm; scale bars, 50 μm. Slides were not counter-stained.

Figure 6 PPAR transcript expressions in the superficial endometrium and trophoblast during early pregnancy. Transcript expression in 1 μg (superficial endometrium) or 250 ng (trophoblast) total RNA was detected by semi-quantitative RT-PCR followed by radioactive hybridization. The histograms represent the means± S.E.M. of the signal normalized to the ACTB value. Data are expressed as percentages of the maximum mean value (a.u., arbitrary units) and are illustrated by representative blots. Different superscripts indicate significant mean differences. LR, data are described by a simple linear regression.
directed towards the nucleus where they can act on nuclear receptors (Lim et al. 1999, Liou et al. 2000).

In the ovine endometrium, the simultaneous presence of the PTGIR membrane receptor (at all stages) and of PTGIS (days 7 to 9) emphasizes the possibility of a transient autocrine role for PGI2, as was previously described in human uterine cells (Battersby et al. 2004). Also, the increase in PTGIR transcript levels in the endometrium on day 17 is concomitant with the up-regulation of PTGIS in the trophoblast, suggesting a potential paracrine role for PGI2 at this time. In the trophoblast, PTGIR transcripts are expressed at day 12 but become undetectable at day 17 when PTGIS is at peak levels, therefore rendering an autocrine role for trophoblastic PGI2 unlikely. These results are at variance with findings in mice, where the PTGIR receptor is present in morulae and blastocysts (Huang et al. 2003) but absent from the uterus at the time of implantation (Lim et al. 1999).

We detected the transcripts for all PPAR and their RXR heterodimerization partners in the ovine endometrium. PPARA, RXRA and RXRG were essentially expressed until day 12 and became virtually undetectable thereafter. After day 12, only PPARD, PPARG and RXRB were expressed. These data were confirmed by localization analysis which revealed that PPARD and PPARG proteins were mainly detected in the luminal epithelium. Unfortunately, RXR protein analysis could not be performed due to the unavailability of cross-reacting antibodies in the ewe. Different heterodimers can therefore form before and after day 12 and carry out different functions. In the trophoblast, PPARD, PPARG, RXRA and RXRG were simultaneously up-regulated after day 12 as development proceeded. The implication of the PPARD/RXRA heterodimer in embryo implantation was previously demonstrated in the mouse, where its activation by PGI2 controls implantation (Lim et al. 1999). The PPARG/RXRA heterodimer is involved in the control of trophoblast invasion in humans (Tarrade et al. 2001). Our study is the first to report the simultaneous expression of PPARD and PPARG transcripts and proteins in a mammalian embryo during the peri-implantation period. It has previously been demonstrated in cellular models (Bastie et al. 2002, Shi et al. 2002) that PPARD regulates the expression of PPARG. It should be noted that in the ovine trophoblast, PPARG expression followed PPARD expression.

Mouse reproduction studies have indicated that PGI2 activates two different pathways. PGI2 originating from the embryo acts on the blastocyst PTGIR receptor to enhance hatching (Huang et al. 2004) and uterine PGI2 acts on uterine PPARD to mediate implantation (Lim et al. 1999). Therefore, in the ewe, PGI2 resulting from the increase in trophoblast PTGIS expression may transduce paracrine signals via the uterine PTGIR receptor and intracrine signals via the trophoblast PPARD receptor.

![Figure 7](image-url) Localization of PPAR protein on representative transversal sections on day-14 pregnant uteri and concepti. GE, glandular epithelium; LE, luminal epithelium; St, stroma; T, trophoblast; En, endoderm; scale bars, 50 μm. Immunohistochemistry with a polyclonal anti-PPARD antibody was revealed with a peroxidase Elite-ABC Vectastain kit and DAB substrate. The controls at day 14 were performed without the first antibody.
However, unlike the mouse model where PGI2-driven implantation signalling is mediated by the uterus only (Lim et al. 1999, Lim & Dey 2000), we suggest that ovine PGJ2 signalling involves not only the uterus (which expresses PTGIR) but also the trophoblast, because it also expresses PTGIS and PPARδ.

To summarize, our results represent a first indication that PGJ2 signalling pathways may contribute to the establishment of pregnancy and the initiation of implantation in ewes. Further studies will be needed to clarify the identity of the uterine and trophoblast genes induced by PGJ2 at implantation.

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