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.

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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 and developmentally 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.* 1999, 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 isoforms are known: PPARA, PPARD and PPARG being encoded by different genes (Desvergnès & Wahli 1999). PPARA was recently identified in the human full-term placenta (Wang *et al.* 2002). However, the knock-out of PPARA in mice produces no reproductive defects (Ding *et al.* 2003). PPARD displays marked induction in the mouse uterus at the time of implantation (Lim *et al.* 1999). The knock-out of PPARD in mice produces placental defects and mid-gestation lethality but no implantation failures (Ding *et al.* 2003). The *PPARG* transcript is expressed in human placental tissues (Marvin *et al.* 2000b) and in bovine embryos (Mohan *et al.* 2002). Knock-out experiments in mice have shown that PPARG regulates terminal differentiation of the trophoblast and that PPARG-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 PGI₂ signalling 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 PGI₂ synthesis and signal transduction. The present study establishes the expression of PGI₂ 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 PGI₂ 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 (decree 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 6 α -methyl-17 α -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 chorionic 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 uterine 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 $\times$ 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 $\times$ 10⁶ c.p.m. was hybridized onto the filters (25 °C for 5 h in 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.01 M Na₂HPO₄, 1 mM Na₂H₂P₂O₇). After washing (25 °C, 2 $\times$ 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 × 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)-dimethylammonio)-1-propane-sulphonate, 1 mM benzamidine-HCl, 1 mM phenylethylsulphonyl and soybean trypsin inhibitor (10 µg/ml), leupeptin (10 µg/ml), aprotinin (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 trophoblast, 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 1:100 000 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.

mRNA species	Primer nucleotide sequence (5' → 3')	GenBank accession number or reference	Species	Ovine PCR fragment size (bp)	Annealing temperature (°C)	Number of cycles
PTGIS	GACCCAGAAATCTACACAGACC ATCAGACCGAAGCCGTACC	NM000961	<i>Homo sapiens</i>	278	56	24–27–30
PTGIR	GTTTCAATGCCTTCAACCC GTTCCCTTTTCCCTC	Z93040	<i>Bos Taurus</i>	200	56	32–35–38
PPARA	GACCTGGAAAGCCGTTATC TGGATGACTCCTCGGCGCTG	AF229356	<i>Bos Taurus</i>	233	59	32–35–38
PPARD	CAGAAGAAGAACCGCAACA CGCCATACCTTGAGAAGGGT	Wang <i>et al.</i> 2002	<i>Homo sapiens</i>	503	50	27–30–33
PPARG	GACAAGGGAGTTTCTAAAG TTTCTGGAGCAGCTTGGCA	NM_005037	<i>Homo sapiens</i>	271	50	32–35–38
RXRA	GGACACCAAACATTTCTGCC GATGTGCTTGGTGAAGGAAGCC	Mohan <i>et al.</i> 2002	<i>Bos Taurus</i>	415	64	32–35–38
RXRB	GGCTGGCAAACGGCTA TGGCCAGGCACTTCTG	Mohan <i>et al.</i> 2002	<i>Bos Taurus</i>	207	50	32–35–38
RXRG	AGTACACAGATACCCCA CACATTCTGCCTCACTCT	Wang <i>et al.</i> 2002	<i>Homo sapiens</i>	550	55	27–30–33
ACTB	GACTACCTCATGAAGATCCTC CGGATGTCCACGTCACACTTC	U39357	<i>Ovis aries</i>	310	57	22–25–28

using the Proview 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 desiccant at 4°C for no longer than 4 weeks. Slides were delipidized for 5 min with chloroform and then re-hydrated in PBS. 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 using an 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.

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 peroxidase 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 second 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 Aqaperm Mounting medium (CML, Nemours, France) and Entellan-mounting medium (VWR, Fontenay sous Bois, France). Photomicroscopy was performed as described previously for ISH.

Statistical analysis

Results were analysed using one-way ANOVA (Systat software GMBH, 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*, *PPARD*, *PPARG* and *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% (*PTGIR*), 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

Figure 1 Detection at the expected size of transcripts for *PTGIS*, *PTGIR*, *PPARA*, *PPARG*, *RXRA*, *RXRB* and *RXRG* in control ovine tissues (kidney for *PTGIS*, *PTGIR* and *PPARG*; liver for *PPARA*, *RXRA* and *RXRB*; cornea for *PPARG*; retina for *RXRG*). Products of RT-PCR were resolved in 2% (w/v) Tris-borate-EDTA-agarose gels and visualized by ethidium bromide staining.

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

Figure 2 Western analysis of *PTGIS* expression in ovine protein extracts. A, blot incubated without anti-*PTGIS* and with the second antibody. B, blot incubated with anti-*PTGIS* and the second antibody. Lanes 1, 5 and 6, 5 µg of a day-14 conceptus extract; lanes 2 and 7, 30 µg of a superficial uterus extract on day 9 of pregnancy; lane 3, protein ladder; lane 4, 5 µg of an endothelium extract. The antibody revealed a unique band at 56 kDa.

($P < 0.05$). 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

Figure 3 *PTGIS* and *PTGIR* expression in the superficial endometrium and trophoblast during early pregnancy. (A) *PTGIS* 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 $\pm$ 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. (B) Western-blot analysis of *PTGIS* protein expression in the superficial endometrium (30 µg/lane) and in the trophoblast (5 µg/lane). The histograms represent the means $\pm$ S.E.M. of the signal produced by 1 µg protein extract normalized to the signal produced by 1 µg artery protein used as a standard, and are illustrated by representative blots. (C) *PTGIR* transcript expression in 1 µg total RNA was detected by semi-quantitative RT-PCR followed by radioactive hybridization. The histograms represent the means $\pm$ S.E.M. of the signal normalized to the *ACTB* value. Data are expressed as percentages of the maximum mean value and are illustrated by representative blots. Different superscripts indicate significant mean differences. LR, data are described by a simple linear regression; ND, not determined.

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, *PPARA* 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. *PPARG* 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

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 μ m. (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.

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

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 $\pm$ 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.

(Rupnow *et al.* 2002). A similar regulation in the endometrium is unlikely because we found that the decrease in PTGIS expression coincided with the known rise in oestradiol 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 7 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 μ m. 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.

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 un-

availability 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 8 RXR transcript expression 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 $\pm$ S.E.M. of the signal normalized to the ACTB value. Data are expressed as percentages of the maximal mean value (a.u., arbitrary units) and are illustrated by representative blots. Different superscripts indicate significant mean differences.

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 PGI₂ signalling involves not only the uterus (which expresses PTGIR) but also the trophoblast, because it also expresses PTGIS and PPARD.

To summarize, our results represent a first indication that PGI₂ 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 PGI₂ at implantation.

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