Localization of prostaglandin synthase type-1 (PGHS-1) mRNA and prostaglandin synthase type-2 (PGHS-2) mRNA in ovine myometrium and endometrium throughout gestation

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Abstract

Increased prostaglandin production by tissues in the sheep uterus and placenta are thought to be important for the onset of parturition. In the sheep placenta, this is most likely due to increased expression of prostaglandin synthase type-2 (PGHS-2) rather than prostaglandin synthase type-1 (PGHS-1). However, there is no information concerning expression of PGHS isoenzymes in maternal uterine tissues during pregnancy. Therefore, the purpose of the present study was to examine the expression of PGHS-1 and PGHS-2 in the sheep myometrium and endometrium during late gestation using in situ hybridization and immunohistochemistry. Using 35S-labelled oligonucleotide probes, which give specific hybridization signals in other tissues, we localized PGHS-2 mRNA to endometrial epithelium, and apparently to other cells in both endometrium and myometrium. This artefactual signal was still present with 100-fold excess unlabelled oligonucleotide probe and with sense probes, but was resolved with the use of ³³P-oligonucleotides. Using ³³P-labelled oligonucleotide probes we could not detect either PGHS-1 or PGHS-2 mRNA in myometrium, and found expression only of PGHS-2 mRNA in endometrium. PGHS-2 mRNA localized to the endometrial epithelium and was undetectable in glandular epithelium. The level of PGHS-2 expression rose significantly between days 80 and 85 of pregnancy and term, and this corresponded to the appearance of immunoreactive PGHS-2 protein, measured by immunohistochemistry, in the endometrial epithelium. Therefore we conclude that ³³P-labelled probes are preferred for detection of mRNAs encoding PGHS-2 in ovine uterine tissues. Expression of PGHS-2 mRNA is greater than that of PGHS-1, increases during gestation, and predominates in the endometrial epithelium, consistent with the site of PGHS-2 protein localization.

Introduction

Prostaglandin (PG) formation by intrauterine tissues appears to play an important role in parturition in a number of different species (Challis & Olson 1988). A key enzyme in the formation of PGs is PGH synthase (PGHS), which catalyses the formation of PGH₂ from arachidonic acid. The PGH₂ so formed can then be converted by various enzymes to PGE₂, PGF₂α, thromboxane, or prostacyclin. Recent studies have demonstrated that PGHS is present as two isoforms. PGHS-1 is constitutively expressed, and its gene possesses a number of similarities to other previously characterized housekeeping genes (Wang et al. 1993). PGHS-2 is an inducible form of the enzyme, and its expression can be increased markedly by a number of substances including growth factors and cytokines (Kujubu et al. 1991, Wong & Richards 1991, Xie et al. 1991, Hla & Neilson 1992, O’Banion et al. 1992, Smith 1992).

In sheep, there is an increase in the concentration of PGE₂ and PGF₂α in fetal and maternal plasma with advancing gestation (Challis et al. 1976, Thorburn & Rice 1990). These PGs are thought to originate from the placenta and fetal membranes (Liggins & Grieves 1971, Evans et al. 1983, Risbridger et al. 1985, Rice et al. 1988, Langlois et al. 1993, Wimsatt et al. 1993, Rice et al. 1995). There is a corresponding increase in PGHS activity (Rice et al. 1988) and protein (Wimsatt et al. 1993) in sheep placenta with advancing gestation, and this appears to be due to increased expression of PGHS-2 mRNA and protein (Rice et al. 1995) rather than the PGHS-1 isomer. Expression of PGHS-2 mRNA occurs predominantly in
mononuclear trophoblasts in the fetal placenta rather than the maternal syncytiotrophoblast (Gibb et al. 1996) and is considered the major step leading to the progressive rise in PGE$_2$ concentrations in the fetal circulation (Challis et al. 1976, Gyomerey et al. 1999).

The principal prostaglandin released by the uterus into the maternal circulation of pregnant sheep is PGF$_{2\alpha}$, and its output increases later than that of PGE$_2$, in association with the time of labour itself (Gyomorey et al. 1976, Gyomorey et al. 1999). The cell type responsible for production of this prostaglandin is unclear, although it has been suggested that maternal glandular cells may be involved in its production. However, the distribution of PGHS iso-enzymes in the endometrium and myometrium of sheep during pregnancy is not known. Oestradiol and progesterone have been shown to regulate the expression of PGHS in the non-pregnant sheep myometrium and endometrium (Wu et al. 1996a). In addition, oestrogen receptors together with heat shock proteins HSP 70 and HSP 90 have been found in the myometrium and endometrium of pregnant sheep. Moreover, PGHS-2 protein and mRNA have been reported to increase in the endometrium and myometrium during spontaneous term or glucocorticoid-induced preterm labour (Zhang et al. 1991).

The purpose of the present study was to localize and quantify the expression of PGHS-1 and PGHS-2 mRNA in sheep endometrium and myometrium throughout pregnancy. In previous studies with ovine placenta and other fetal tissues we had utilized $^{35}$S-labelled oligonucleotide probes for in situ hybridization. We present evidence that these are unsuitable for in situ hybridization studies of PGHS expression in the ovine uterus, but with $^{33}$P-labelled probes, the localization of PGHS mRNAs can be easily described.

Materials and Methods

Animals and tissues

Pregnant ewes of mixed age and breeds and of known gestational age (day of natural mating = Day 0) were used in these studies. The ewes were killed with an overdose of nembutal (Euthanyl; Abbott Laboratories, Montreal, PQ, Canada) at various times during pregnancy. Uteri from three animals at each gestational age group were examined (80–87 days, 120–127 days and term (term is approximately 145–147 days)). Tissues were frozen on dry ice, sectioned (15 µm) by use of a cryostat, fixed briefly in paraformaldehyde, dehydrated through a graded ethanol series, and stored in 95% ethanol at 4 °C.

Oligonucleotide probes

Sense and antisense oligonucleotide probes were synthesized by the core molecular biology facility of the University of Ottawa with an Oligo 1000 DNA synthesizer (Beckman Instrument Inc., Mississauga, ON, Canada) and were based upon the published structures of human PGHS-1 and PGHS-2 (Yokoyama & Tanabe 1989, Hla & Neilson 1992). The sequence of the probe for PGHS-1 was GTG GCC GTC TTG ACA ATG TTA AAG CCC ACC TCG CCG CCA AAT GTG CTC GG, which corresponded to nucleotides 1641–1690 of human PGHS-1. The sequence for the probe to PGHS-2 was GGG ACA GCC CTT CAC GTT ATT GCA GAG AGA CTG AAT TGA GGC AGT GT, which corresponded to nucleotides 1734–1783 of human PGHS-2. By use of the Bstnl 1·4·6 MP program (Altschul et al. 1990), it was shown that the probe to PGHS-1 had 93% homology with the corresponding region of the sheep PGHS-1 cDNA, which has been cloned (DeWitt & Smith 1990).

In situ hybridization

The method for in situ hybridization has been described previously in detail (Matthews et al. 1991). Briefly, the slides were removed from the ethanol, allowed to air dry at room temperature, and then incubated overnight in a moist chamber at 42 °C with the radio-labelled oligonucleotide probe in hybridization buffer. The hybridization buffer used for these experiments was prepared in our laboratory with materials from BDH (Toronto, ON, Canada) and Sigma Chemical Co. (St Louis, MO, USA) unless noted otherwise. It contained 4-strength SSC (single strength SSC is 150 mM sodium chloride, 15 mM sodium citrate), 50% deionized formamide (Gibco BRL, Burlington, ON, Canada), 50 mM sodium phosphate (pH 7·0), 1 mM sodium pyrophosphate (pH 7·0), 0·02% BSA (Boehringer Mannheim, Dorval, PQ, Canada), and 40 mM dithiothreitol. The oligonucleotide probes were labelled by use of terminal deoxynucleotidyl transferase (Gibco BRL) with $^{35}$S-labelled deoxyadenosine 5′- (α-thio) triphosphate (1300 Ci/mmole) or $^{35}$P-labelled deoxyadenosine 5′ triphosphate (2000 Ci/mmole; NEN, DuPont Canada Inc., Mississauga, ON, Canada). Probes were purified using Nensorb 20 columns and were used at a concentration of 5000 c.p.m./μl. Labelled probe in hybridization buffer (200 μl) was applied to each slide and incubated overnight at 45 °C in a moist chamber. After washing for 20 min in single-strength SSC at room temperature and for 45 min in single strength SSC at 55 °C, the sections were rinsed in single-strength SSC and 0·1-strength SSC (10 s each), dehydrated in ethanol, dried, exposed to X-ray film (Biomax; Eastman Kodak, Rochester, NY, USA) and then dipped in Ilford K5 (Mobberley, UK) liquid emulsion. The slides were then exposed to X-ray film for 5 days and emulsion-coated sections were incubated for 28 days. They were then developed according to standard procedures. The sections
were counterstained with cresyl violet to permit identification of nuclei.

**Immunohistochemistry**

Sections were processed for immunohistochemistry with the use of an Elite Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Deparaffinized sections were incubated in 3% H$_2$O$_2$ in methanol for 20 min to quench endogenous peroxidase activity and washed in PBS for 10 min. PBS was prepared in our laboratory and was composed of monosodium dihydrogen phosphate (0.2 g/l), disodium hydrogen phosphate (1.375 g/l) and sodium chloride (8.8 g/l) adjusted to pH 7.5 (all chemicals from BDH). Sections were then incubated with 10% normal goat serum (Elite Vectastain ABC Kit) in PBS for 30 min to block non-specific binding. The primary polyclonal antibody to human PGHS-2 raised in rabbits (PG27; Oxford Biochemical Research Inc., Oxford, MI, USA) was diluted in PBS containing 1% BSA and incubated with the tissues at 4 °C for 18–20 h. Slides were then brought to room temperature, washed in PBS (2 × 5 min), incubated with biotinylated second antibody for 1 h and washed in PBS (3 × 5 min). Avidin–biotin–peroxidase complex in PBS was applied for 1 h. After washing in PBS (3 × 5 min), the sections were incubated in diaminobenzidine tetrahydrochloride (DAB) in PBS with 0.01% hydrogen peroxide for 4 min. The sections were counterstained with Carrazi’s haematoxylin, dehydrated through a graded series of ethanols, cleared in xylene, and mounted with Permount (Fisher Scientific, Nepean, ON, Canada). For control incubations, the tissues were incubated with the appropriate dilution of non-immune antisera (PG2OC; Oxford Biomedical Research Inc.).

**Quantification**

In each experiment, all control and experimental sections were processed simultaneously to allow direct comparison between groups. For in situ hybridization, the sections were exposed together with $^{14}$C standards (Amersham Life Sciences, Little Chalfont, UK) to ensure analysis in the linear region of the autoradiographic film. The relative optical density of the signal on the autoradiographic film was quantified, after subtraction of background values.

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**Figure 1** Emulsion autoradiography of term pregnant sheep uterus using $^{35}$S-labelled oligonucleotide probes for PGHS-1 and PGHS-2. LE, luminal epithelium; GE, glandular epithelium; M, myometrium. (a) Antisense PGHS-1 probe, (b) sense PGHS-1 probe, (c) antisense PGHS-2 probe, (d) sense PGHS-2 probe. Magnification bars: 50 μm.
through use of a computerized image analysis system (Imaging Research, St Catherines, ON, Canada). The value obtained represents an average density over the area measured. Comparison between groups was performed using the mean values obtained from three sections per animal.

**Statistical analysis**

Results are presented as mean ± s.e. and were analysed by ANOVA followed by Scheffé Multiple Comparison test.

**Results**

We have previously used 35S-oligonucleotide probes to localize PGHS mRNA and other mRNAs in ovine placenta (Gibb et al. 1996) and other tissues (Matthews et al. 1991). This method proved unsuccessful with the sheep uterus. When 35S-labelled probes were used, PGHS-2 mRNA appeared to be localized strongly to the luminal epithelium of the endometrium and also to endometrial stroma, and myometrium (Fig. 1). PGHS-1 mRNA appeared to be absent from the luminal epithelium of the endometrium but had a similar distribution to PGHS-2 mRNA in the endometrial stroma and myometrium. These signals also appeared to be strongly localized to specific cells. However, when sense probes were used as controls, the apparent expression for PGHS-1 and PGHS-2 mRNA in myometrium and endometrial stroma was similar. In addition, increased, rather than decreased, expression was seen when 100-fold excess of unlabelled probe was added as a control (data not shown). This pattern persisted in uterine tissues obtained throughout the course of gestation.

When 33P-labelled probes were examined (Fig. 2) we obtained a different pattern of specific hybridization. The PGHS-2 mRNA was indeed localized to the luminal epithelium of the endometrium, but was not detected in the endometrial stroma, glands or the myometrium. Addition of 33P-labelled sense probe (Fig. 2b,d) or 100-fold excess of non-radioactive probe (data not shown) did not result in any signal. No evidence for PGHS-1 mRNA expression was found in any of the uterine tissues (Fig. 2a). PGHS-2 protein was also localized principally to the luminal epithelium and was undetectable or at low levels in the endometrial stroma and glands (Fig. 3).

The expression of PGHS-2 mRNA was examined throughout gestation using 33P-labelled probes (Fig. 4). PGHS-2 mRNA was localized to the luminal epithelium of the endometrium at all gestational ages. Regression analysis of the relative optical density versus gestational age demonstrated a significant increase in expression occurred from 80–85 days to term ($r=0.91, P<0.006$).

Discussion

PGHS has an important role in the regulation of prostaglandin synthesis in intrauterine tissues at parturition in human and ovine pregnancy (Challis et al. 1997). In particular the PGHS-2 isoform rather than the PGHS-1 isoform of the enzyme appears to be upregulated in human fetal membranes (Hirst et al. 1995, Mijovic et al. 1997) and sheep placenta at parturition (Wimsatt et al. 1993, Gibb et al. 1996). In the present study we have shown that in ovine endometrium PGHS-2 mRNA is preferentially expressed compared with PGHS-1 mRNA and is localized to the luminal epithelium rather than the glandular epithelium and stroma. During gestation there is increased expression of this isoform in the endometrium and expression in the endometrium is greater than that in the myometrium. In addition, we have used 33P-labelled oligonucleotide probes instead of 35S-labelled oligonucleotide probes to study PGHS-2 expression in the endometrium and myometrium as 35S-labelled probes were not found to be suitable for studying these intrauterine tissues.

Previous studies with the ovine placenta (Gibb et al. 1996), brain (Matthews et al. 1991) human fetal membranes (Gibb & Sun 1996) and other tissues have demonstrated clearly the feasibility of using 35S-labelled oligonucleotide probes to localize various species of mRNA. In the present study with ovine uterus, however, 35S-labelled PGHS probes were apparently unsuitable. Although 35S-labelled probes did localize the mRNA to the luminal epithelium (Fig. 1) there was additional non-specific binding in the endometrial stroma and throughout the myometrium. In the latter report, the labelling procedure and methodologies are different from those we have used with

Figure 3 Immunohistochemical localization of PGHS-2 in ovine uterus. (A) PGHS-2 is present in luminal epithelium (LE), (B) non-immune control. Tissue shown, 130 day gestation. Magnification bars: 10 μm.
oligonucleotide probes, and the present problems that we encountered are not necessarily extrapolated to these studies. However, the present findings do highlight potential problems performing in situ hybridization with 35S-labelled probes, which appear to be tissue specific.

Using 33P-labelled oligonucleotide probes we have shown clearly that PGHS-2 mRNA expression predominates over that of PGHS-1 mRNA in the ovine endometrium during pregnancy. The PGHS-2 mRNA was localized to the luminal epithelium and was undetectable in the endometrial stroma, glands and the myometrium.

Previously it has been suggested that PGHS-2 protein localized to the glandular epithelium in endometrium from non-pregnant (Wu et al. 1996b) or pregnant (Zhang et al. 1996) animals. In the present study, PGHS-2 protein and mRNA were confined to the luminal epithelium and were consistent with previous studies indicating that PGHS-2 mRNA and protein were at low levels (or absent) from the endometrial stroma (Wu et al. 1996b, Zhang et al. 1996). It is possible that luminal epithelium was described wrongly as glandular epithelium in sections where it appears to be separate from the lumen, in some

Figure 4 Localization of PGHS-2 mRNA in sheep uterus throughout gestation. A 33P-labelled oligonucleotide probe was used. Emulsion autoradiography of typical uterine tissues obtained at 80–85 days, 120–127 days and term. Magnification bars: a, c, e: 25 μm; b, d, f: 10 μm.
previous reports. Our failure to detect mRNA encoding either PGHS isoform in myometrium is surprising since immunoreactive PGHS-2 has been identified with this tissue (Wu et al. 1996b, Gyomorey et al. 1999), and PGHS expression is well established in the myometrium of other species (Myatt et al. 1994, Moore et al. 1999). It is therefore likely that levels of steady-state mRNA encoding one or both PGHS enzymes are very low in myometrium from pregnant sheep, and certainly much lower than in the endometrial epithelium.

The present results, taken in conjunction with previous studies on the ovine placenta, suggest that there is specific upregulation of the PGHS-2 isomer of PGHS in compartments of the ovine uterus during gestation. In the endometrium PGHS-2 is localized almost exclusively to the luminal epithelium while in the placenta it is localized to the trophoblast mononuclear cells. In the human, glucocorticoids can stimulate PGHS-2 expression in amnion cells (Economopoulos et al. 1996) and in mixed cultures of chorion (Whittle et al. 1999). Glucocorticoid concentrations increase in the plasma of fetal sheep during the latter part of gestation, and it is possible that cortisol may be the direct stimulus for increased PGHS-2 expression in the trophoblast layer of the sheep placenta. The factors responsible for increasing levels of PGHS-2 mRNA in the endometrium are not known, but could also include glucocorticoids and/or oestrogen (Wu et al. 1996a). The changes described in levels of endometrial PGHS-2 mRNA with time of pregnancy are consistent with the proposition that this tissue may be the source of the prepartum increase in maternal PGF_{2\alpha} although resolution of this question will require more frequent and precise sampling times during the course of the labour process.

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References


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