Introduction

The ovine placenta is a major source of prostaglandin production at term delivery in ewes. Rice et al. (1988) demonstrated that ovine placental microsome preparations show increased cyclooxygenase activity during the last 30 days of pregnancy. Evans et al. (1982) showed that preparations of dispersed placental cells have an increased capacity to produce PGE$_2$, PGF$_2\alpha$, and 6-keto-PGF$_1\alpha$ from endogenous precursors with the progression of gestation. Furthermore, Risbridger et al. (1985) demonstrated that the capacity of ovine trophoblast cells to synthesize PGs in vitro is low during early and mid-gestation and increases only after day 100 of pregnancy.

Boshier et al. (1991) were the first investigators to document the ontogenic profile and cellular localization of the prostaglandin G/H synthase (PGHS) enzyme in sheep placental tissue using immunohistochemistry. In placentomes from early pregnancy (days 30–54), immunoreactive PGHS was present in the mononuclear trophoblast cells of the fetal placenta. Cells in the maternal mesenchyme and epithelial syncytium were weakly immunopositive for this enzyme. PGHS-1 immunoreactivity was also demonstrated in the endothelial cells of the chorionic vessels. The PGHS-2 isozyme was localized exclusively to the trophoblast epithelial cells. Immunoreactive PGHS-2 was not detectable in the maternal epithelial syncytium or the stroma of the cotyledons. The binucleate cells of the fetal placenta were consistently immunonegative for both PGHS isozymes. These results indicate that the cellular localization of PGHS-1 and PGHS-2 in ovine placenta does not change during the last 15 days of pregnancy. Co-localization of these isozymes indicates that the source of arachidonic acid and the site of prostanoid formation are the same. Quantitation of the percentage area of positive staining for PGHS-1 and PGHS-2 using image analysis software demonstrated a significant increase in PGHS-2 in the fetal trophoblast after glucocorticoid-induced labour and spontaneous parturition. This finding indicates that increased formation of the PGHS-2 isozyme is responsible for the large increase in prostaglandin production by the ovine placenta at term labour.
immunohistochemistry revealed an increase in the content of PGHS-2 in mononuclear trophoblast cells of the placenta after day 140 of gestation. It was concluded that PGHS-2 is the enzyme responsible for placental prostaglandin production at term delivery. However, the study of Gibb et al. (1996) did not elucidate the cellular localization of PGHS-1 expression nor did it address the factors that regulate enzyme formation.

Glucocorticoids regulate prostanoid production in many tissues. PGHS activity increases after glucocorticoid treatment in mouse myeloid leukaemia (MI) cells (Honma et al., 1980), fetal rat lungs (Tsai et al., 1983) and in Swiss 3T3 mouse fibroblast cells (Chandrabose et al., 1980). Human amnion cells in primary culture have an increased capacity to convert exogenous arachidonic acid into PGE$_2$ in response to glucocorticoid injection when labour was established. Labour onset in ewes is preceded by activation of the fetal hypothalamic-pituitary-adrenal axis (HPA axis), it is possible that the prepartum increase in fetal cortisol production acts to modulate intrauterine PGHS expression.

McLaren et al. (1996) demonstrated that the placentome is the principal site of induced PGHS expression at the time of glucocorticoid-induced labour in sheep, although the distribution of the two forms of the enzyme within the placentome was not addressed. The aim of the present study was to characterize the cellular distribution of ir-PGHS-1 and ir-PGHS-2 in ovine placental tissue using immunohistochemical staining. The primary sites of enzyme production were determined in placentomes after glucocorticoid-induced and spontaneous labour and were compared with those of ewes in late gestation but not in labour.

**Materials and Methods**

**Animals**

Sixteen pregnant Border Leicester–Merino crossbred ewes of known gestational age were used in this study. The animal experiments were approved by the Monash University Standing Committee on Ethics in animal experimentation. Ewes underwent surgery between 118 and 125 days of gestation to implant electromyogram electrodes. Electrodes were implanted into the myometrium to enable labour onset to be identified (Harding et al., 1982).

A paired treatment-control experimental paradigm was used in the first part of the study to determine the effects of glucocorticoid on PGHS expression. Fetal sheep were injected using guided ultrasonography (via maternal transabdominal injection) with either glucocorticoid (betamethasone; Celestone Chronodose, Schering Plough Pharmaceuticals, Baulkham Hills, NSW; 5.7 mg ml$^{-1}$ in 1 ml total volume; $n = 5$) or an equivalent volume of sterile isotonic saline (control; $n = 5$) on day 131 of gestation. Animals that received betamethasone were killed by i.v. barbiturate injection when labour was established. Labour onset occurred 56.6 ± 0.8 h after glucocorticoid injection and was determined from increased uterine electromyogram activity and clinical factors such as swelling of the maternal udder and vulva. Control ewes treated with saline were killed at the same time as the experimental ewes to obtain age-matched control tissues. The procedure of intrafetal injection of glucocorticoid has been characterized and validated (McLaren et al., 1996).

Six sheep were used for the second part of the study to examine PGHS-1 and PGHS-2 expression at spontaneous-onset labour. Ewes were allowed to progress to term and were killed by i.v. barbiturate injection when increased uterine contractile activity (consistent with labour onset) had been observed for 6–8 h. The mean age to labour onset in these ewes was 149 days.

**Tissue collection**

After the animals were killed, a post mortem examination was conducted and tissues were collected in the shortest possible period. Placentomes (sliced vertically along a radial axis of symmetry) were placed in cold Bouin’s fixative solution for 24 h. Tissues were washed five times in 70% ethanol before dehydration and embedding. Tissues were dehydrated using an automatic tissue processor (Histokinette, Thomas Optical and Scientific Co. Pty Ltd). Tissues were incubated sequentially in 70%, 90% and 100% ethanol for 12 h (2 × 2 h washes), cleared of dehydrating agents by washing in Histosol (2 × 2 h washes) and embedded in paraffin wax. Tissue sections (7 µm) were prepared by standard techniques and placed on glass slides coated with poly-L-lysine (0.01%; Sigma Chemical Company, St Louis, MO).

**Immunohistochemistry**

Immunohistochemistry was performed using the DAKO ENVISION™ System (DAKO Corporation, Carpinteria, CA). After paraffin wax was removed from the tissue sections, they were incubated with 0.03% (w/v) $\text{H}_2\text{O}_2$ for 5 min to quench endogenous peroxidase activity. Tissue sections were incubated for 1 h at room temperature with PGHS-1 or PGHS-2 antibody at 1:1000 dilution. PGHS-1 antiserum was purchased from the Oxford Biochemicals Company (Oxford, MI). The crossreactivity of the antibody with the PGHS-2 isozyme was estimated to be < 0.1%, as determined from laser densitometry (McLaren et al., 1996). The polyclonal PGHS-2 antiserum, raised in rabbits against rat seminal vesicle PGHS (McLaren et al., 1996). PGHS used for injection (99% purity) was purchased from the Oxford Biochemicals Company (Oxford, MI). The crossreactivity of the antibody with the PGHS-2 isozyme was estimated to be < 0.1%, as determined from laser densitometry (McLaren et al., 1996). The polyclonal PGHS-2 antiserum, raised in rabbits against murine PGHS-2, was purchased from the Cayman Chemical Company (Ann Arbor, MI). There was no detectable crossreactivity of the antibody with the PGHS-2 isozyme was estimated to be < 0.1%, as determined from laser densitometry (McLaren et al., 1996). After washing with Tris-buffered saline (TBS) (pH 7.5), the sections were incubated with peroxidase-labelled polymer conjugated to goat anti-rabbit IgG second antibody for 30 min. Slides were rinsed once and the substrate–chromogen solution was added for 10 min. Specific immunostaining was identified using diaminobenzidine. The sections were counterstained with Harris’
haematoxylin, dehydrated and mounted with DPX moun-
tant. Immunoreactivity in tissue sections was examined
using a Leitz Wetzlar Dialux 20 microscope.

Controls

Tissues from animals of each group were processed
simultaneously to allow direct comparison between staining
runs. The following negative controls (derived from the
same specimen) were included in every staining run to
monitor daily variations in the immunohistochemical
staining procedure and to verify the performance of the
reagents: (i) the PGHS-1 or PGHS-2 primary antibody
was substituted either by antibody dilution buffer or by non-
immune rabbit serum (1:1000 dilution); (ii) the peroxidase-
labelled secondary link antibody (goat anti-rabbit immuno-
globulins) was substituted with TBS (pH 7.5) wash buffer;
and (iii) the slide section was only incubated with TBS
(pH 7.5) diluent before the addition of the substrate-
chromogen solution.

If any of the control specimens demonstrated residual
background staining, the results of the staining run were
considered to be invalid.

Pre-absorption controls were also performed to verify that
the staining observed in tissue sections was due to binding of
the primary antibody to its target antigen. PGHS-2 antibody
(protein concentration: 33 μg ml⁻¹) was pre-absorbed with an
excess (4000 times greater protein concentration) of PGHS-2
antigen (protein concentration: 1.04 μg ml⁻¹) for 18 h at 4°C.
Similarly, the PGHS-1 antibody (protein concentration: 83 μg
ml⁻¹) was pre-absorbed with an excess (4000 times greater
protein concentration) of PGHS-1 antigen (protein concen-
tration: 1 μg ml⁻¹) for 18 h at 4°C.

After addition of precipitating solution (0.05 mol phos-
phate buffer 1⁻¹, 0.25% BSA, 5% polyethylene glycol 6000,
0.125% (v/v) normal rabbit serum and 0.5% (v/v) goat anti-
rabbit IgGs), the mixtures were centrifuged at 1200 g for
30 min at 4°C. The supernatant was incubated with the tissue
section. The tissue sections used for the PGHS-1 and PGHS-2
pre-absorption control incubations were derived from a
betamethasone-injected animal in labour previously shown
to demonstrate positive staining for both isozymes. A serial
section of the same specimen was run concurrently with
every pre-absorbed antibody control tissue to act as a
positive control.

Quantitation of immunohistochemical staining

Quantitative analysis of positive staining for ir-PGHS-1
and ir-PGHS-2 in placental tissue sections was performed
using image analysis software (Zeiss KS 400, Version 3.0).
Five placentomes collected from the different animals within
each treatment group (saline injection and betamethasone
administration) and six placentomes collected from the
animals in the spontaneous labour group were examined for
immunoreactivity. The percentage area of positive staining
was calculated from five fields of view (× 20 magnification)
for each tissue section examined. Two sections per placen-
tome were assessed for the degree of positive staining. The
results are expressed as the mean percentage area of positive
staining for PGHS-1 and PGHS-2.

Statistical analysis

Statistical analysis of the data was performed using a
commercially available statistical program (GraphPad Prism
Version 2.01). Data were first analysed by a univariate
homogeneity of variance test (Bartlett’s Box F test). If
significance was found for a particular parameter (that is the
raw data were non-homogeneous), the test was repeated
using log₁₀ transformation and square root transformation.
The transform most closely attaining homogeneity was then
used for all subsequent statistical analyses. Significant
interactions occurring between two or more factors were
identified using multifactorial ANOVA. The post hoc test of
least significant difference (LSD) was used subsequent to
the ANOVA to identify significant differences between pairs
of mean values. A probability level of 5% (P < 0.05) was
specified as significant. The data are presented graphically in
an untransformed state. Values are expressed as mean ± SEM.

Results

PGHS-1 immunoreactivity

The location and the percentage area of tissue expressing
PGHS-1 immunoreactivity in sheep placentomes obtained
after intrafetal saline injection, glucocorticoid-induced
labour and spontaneous parturition are shown (Figs 1
and 2, respectively). In all groups studied, PGHS-1 im-
munostaining was localized to mononuclear cells of the
trophoblastic epithelium. The binucleate cells were clearly
immunonegative. The endothelial cells of fetal vessels in the
chorionic villi demonstrated weak immunostaining (Fig. 1b,
arrow). Moreover, the fibroblasts of the fetal stroma were
weakly immunopositive for PGHS-1. Both the fetal and
maternal mesenchymal core demonstrated diffuse staining
for PGHS-1. There was no significant effect of spontaneous
parturition or treatment (saline injection or glucocorticoid-
induced labour) on the location and the percentage area of
positive staining for PGHS-1 in placental tissue sections.

When control incubations were performed with primary
PGHS-1 antibody that had been pre-absorbed with purified
PGHS-1 isolated from ram seminal vesicles, no staining
was observed (Fig. 3a). Similarly, no staining for ir-PGHS-1 was
observed when the primary antibody was substituted in the
staining procedure with normal non-immune rabbit serum
(Fig. 3b).

PGHS-2 immunoreactivity

PGHS-2 was present in placental cotyledon tissue sections
on day 133 of gestation, as demonstrated in the saline-
 injected control animals (Fig. 4a). Staining was confined to
the trophoblastic mononuclear epithelial cells. Binucleate
cells were consistently immunonegative. In contrast to the PGHS-1 isozyme, the endothelial cells of fetal vessels in the chorionic villi and the fibroblasts of the fetal stroma did not contain ir-PGHS-2. Furthermore, both the fetal and maternal stroma were immunonegative for PGHS-2. The location of staining for PGHS-2 did not alter in response to glucocorticoid-induced (Fig. 4b) or spontaneous labour (Fig. 4c); however, there was a significant increase in the percentage area of positive staining for PGHS-2 at labour onset (Fig. 2; \( P < 0.05 \)).

The amount of positive staining for PGHS-2 in placental tissue collected after glucocorticoid-induced labour and spontaneous parturition was significantly higher than that after intrafetal saline injection.

When control incubations were performed with primary PGHS-2 antibody that had been pre-absorbed overnight with purified PGHS-2 isolated from sheep placenta (70% purity), no staining was observed in cells that previously demonstrated positive staining for the PGHS-2 isozyme. Moreover, no positive immunoreactivity was observed in tissue sections in which the primary antibody was substituted with non-immune rabbit serum (Fig. 3d).

**Discussion**

This study has demonstrated the presence and localization of ir-PGHS-1 and ir-PGHS-2 in sheep placental tissue after glucocorticoid-induced labour and spontaneous parturition. Animals administered an intrafetal injection of isotonic saline on day 131 of gestation acted as non-labour controls. In all groups studied, the PGHS-2 isozyme was localized exclusively to trophoblastic epithelial cells; no detectable
staining was observed in the intervening maternal epithelium or stroma of the cotyledons. Similarly, ir-PGHS-1 was present primarily in the trophoblast cells, although cells in the maternal mesenchyme and epithelium were weakly immunopositive for this enzyme. There were no cell-specific alterations in PGHS-1 or PGHS-2 immunoreactivity in sheep placentomes during the last 10–15 days of pregnancy.

Furthermore, localization of enzyme staining was not influenced by labour-onset, whether spontaneous or glucocorticoid-induced.

The placenta has been identified as the major site of prostaglandin production in the uterus of pregnant sheep. Risbridger et al. (1985) demonstrated that prostaglandin synthesis by dispersed ovine cotyledonary cells is low during early and mid-gestation but increases markedly from day 110–120 of gestation to term. In that study, exogenous administration of arachidonic acid to incubations of cotyledonary cells did not significantly increase prostaglandin synthesis. This finding is consistent with the suggestion that PGHS activity in placental trophoblast cells increases only after day 110 of gestation. The results of the present study indicate that the fetal trophoblast cells are the primary site of prostaglandin formation during the last 10–15 days of pregnancy.

In the past, studies of placental endocrine function \textit{in vitro} have relied on the use of heterogeneous placental explants (Hoffmann \textit{et al.} 1979; Matt and MacDonald, 1984) or cell suspensions (Branchaud \textit{et al.}, 1983; Shemesh \textit{et al.}, 1984a,b) to elucidate possible sites of hormone formation. Although these studies have contributed to our knowledge of placental endocrinology, the contribution of individual types of cell was not elucidated. In an attempt to delineate the principal sites of prostaglandin output in sheep placenta, Mitchell and Flint (1978) manually separated the fetal cotyledon from the maternal caruncle. The prostaglandin synthesizing capacity of the two sides of the placentome was then investigated using a dispersed cell preparation. Since the fetal cotyledon had a greater prostaglandin output than the maternal uterine epithelium, it was suggested that PGHS activity was primarily localized to the fetal side of the placentome. However, in that study, no anatomical evidence was presented to support the completeness of separation of fetal and

\textbf{Fig. 3.} Control tissue sections for the prostaglandin G/H synthase 1 and 2 (PGHS-1 and PGHS-2) staining procedures. When the primary PGHS-1 antibody was pre-absorbed with purified immunoreactive PGHS-1 (ir-PGHS-1) isolated from ram seminal vesicles (a), no staining was observed. Similarly, when the primary PGHS-2 antibody was pre-absorbed with ir-PGHS-2 isolated from sheep placenta (c), no positive staining was observed. When the primary antibodies were substituted in the immunohistochemical staining procedure with non-immune rabbit serum, no staining was observed (b,d). The tissue used for the control staining procedures was derived from an animal in labour after intrafetal injection of betamethasone. This tissue was previously shown to demonstrate positive staining for the PGHS-1 and PGHS-2 isozymes. BNC: binucleate cell; F: fetal stroma; M: maternal placenta; T: trophoblastic epithelium. Scale bars represent 50 $\mu$m.
maternal components. Given the high degree of interdigitation of the chorionic villi with the crypts of the uterine mucosa, complete separation of the ovine cotyledon into fetal and maternal components is not technically feasible. Thus, the results are potentially confounded by the possibility of a non-homogeneous cell preparation.

Evans et al. (1982) measured PGE$_2$, PGF$_{2\alpha}$ and 6-keto-PGF$_{1\alpha}$ output by sheep cotyledons at different stages of pregnancy using an in vitro cell culture system. The capacity of dispersed placental cells to synthesize these prostaglandins was higher on days 130 and 145 than on days 50 and 100 of gestation. This finding is consistent with the suggestion of increased PGHS expression; however, the localization of enhanced PGHS production could not be characterized as no histological assessment was made of the types of cell in the preparation. Risbridger et al. (1985) used a similar dispersion protocol and suggested that cells of the fetal trophoblast were the major site of prostaglandin production during pregnancy and parturition. Although these investigators demonstrated that enzymatic digestion of the placental tissue yielded binuclear and mononuclear cells, the dispersion technique excluded syncytial cells from the final preparation. Thus, the contribution of maternal epithelial cells to prostaglandin output was not demonstrated.

The data presented in the present study clearly demonstrate that PGHS-1 and PGHS-2 enzyme formation predominantly localizes to the mononuclear cells of the fetal trophoblast. Other studies have confirmed that the subcellular locations of PGHS-1 and PGHS-2 are also the same. PGHS-1 was first shown to be localized to the endoplasmic reticulum and nuclear membrane of kidney tissue sections using immunofluorescence (Smith and Wilkin, 1977; Smith and Bell, 1978). This result was later confirmed by immunoelectron microscopy of cultured mouse fibroblasts (Rollins and Smith, 1980). Regier et al. (1993) demonstrated that PGHS-2 is associated with the endoplasmic reticulum and nuclear envelope of mouse 3T3 fibroblast cells.

Unlike the mononuclear trophoblast cells of the fetal syncytium, binucleate cells of the trophoblast were shown to be immunonegative. This result is consistent with the findings of Boshier et al. (1991) and Gibb et al. (1996) and indicates that these cells are not important to placental prostaglandin formation. Rather, binucleate cells have two alternative functions that are important to the normal progression of pregnancy: (i) to form the fetomaternal syncytium essential for successful implantation and subsequent placental growth; and (ii) to produce and secrete protein and steroid hormones. During the last two-thirds of pregnancy, placental lactogens are measurable in the maternal and fetal circulations of sheep (Chan et al., 1978; Martel and Lacroix, 1978), cattle (Wallace et al., 1985) and goats (Currie et al., 1990). The binucleate cells are the sole source of these hormones. Furthermore, binucleate cells of sheep and cows are capable of considerable progesterone production from endogenous sources (Reimers et al., 1985; Ullman and Reimers, 1989; Wango et al., 1991).

Quantitation of immunoreactive staining in placental tissue sections using image analysis software demonstrated a significant increase in PGHS-2 in association with glucocorticoid-induced and spontaneous-onset labour. This result strongly supports previous reports of increased PGHS-2 protein concentrations in sheep placenta after glucocorticoid-induced labour using western blot analysis.

Fig. 4. Patterns of immunostaining for immunoreactive prostaglandin G/H synthase 2 (ir-PGHS-2) in placental tissue collected from sheep after (a) intrafetal saline injection, (b) glucocorticoid-induced labour and (c) spontaneous labour. PGHS-2 immunoreactivity was localized to mononuclear cells of the trophoblastic epithelium. Binucleate cells and fibroblasts of the fetal stroma were immunonegative. Maternal placental tissue did not express the PGHS-2 isozyme. BNC: binucleate cell; F: fetal stroma; M: maternal placenta; T: trophoblastic epithelium. Scale bar represents 50 μm.
In saline-injected control animals, expression of the PGHS-2 isozyme was low. PGHS-2 staining patterns in placental tissue sections from animals in labour reflect higher enzyme production rates by uninucleate trophoblast cells rather than increased expression by a more diverse group of cells.

In summary, co-localization of PGHS-1 and PGHS-2 implies that the source of arachidonic acid, the site of prostanoid formation and the mechanism of product transport from the inside to the outside of the cell are the same for these two isozymes. The primary difference between PGHS-1 and PGHS-2 lies in the differential regulation of enzyme expression. PGHS-1 isozyme formation is constitutive. Conversely, PGHS-2 expression is induced by a variety of stimulatory factors including glucocorticoid-induced enzymes and cytokines (Liggins and Thorburn, 1994). Regulation of PGHS-2 formation is critical to myometrial activation that results in birth.

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