Localisation and temporal changes in prostaglandin G/H synthase-1 and -2 content in ovine intrauterine tissues in relation to glucocorticoid-induced and spontaneous labour

W J McLaren¹, I R Young² and G E Rice³

¹Department of Pharmacology, Monash University, Clayton, Victoria, Australia

²Department of Physiology, Monash University, Clayton, Victoria, Australia

³Department of Obstetrics and Gynaecology, University of Melbourne, Royal Women's Hospital, Carlton, Victoria, Australia

(Requests for offprints should be addressed to I R Young, Department of Physiology, Monash University, Clayton, Victoria, 3168, Australia;
Email: ross.young@med.monash.edu.au)

Abstract

Parturition in the ewe is preceded by an increase in the synthesis of prostaglandins (PGs) by gestational tissues. To establish the uterine source of these PGs, placental cotyledons, fetal membranes and maternal uterine tissues were collected from ewes ($n=6$) at spontaneous parturition. Solubilised tissue extracts were prepared and analysed by Western blots using polyclonal antibodies to PG G/H synthase-1 and -2 (PGHS-1 and PGHS-2). PGHS-1 was expressed by all intrauterine tissues at term labour. Densitometric analysis of Western blot autoradiographs showed that the fetal membranes and maternal cervix contained the largest amounts of PGHS-1. PGHS-1 enzyme content of ovine amnion was significantly greater than that of either chorion or allantois ($P<0.05$). PGHS-1 protein content of myometrial, endometrial and cotyledonary tissue extracts was minimal. Formation of the PGHS-2 isozyme was confined to placental tissue at term labour. PGHS-2 protein levels in sheep placenta were significantly higher than those of PGHS-1 in all intrauterine tissues examined. This result supports the hypothesis that PGHS-2 is a major contributor to PG formation at term labour.

To elucidate the developmental changes in PGHS-1 and PGHS-2 relative to labour onset, an experimental paradigm of glucocorticoid-induced delivery was used. Previous characterisation and validation of this labour model demonstrated that direct, transabdominal, intrafetal injection of the synthetic glucocorticoid betamethasone

(5.7 mg in 1 ml aqueous vehicle) on day 131 of gestation induced labour onset in 56.6 ± 0.8 h (mean $\pm$ s.e.m.). As the latent period to induced-labour was known, the time course of enzyme formation could be ascertained. Sheep ($n=20$) were killed by barbiturate injection at various time intervals post-injection (0, 14, 28, 42 and 56 h). Tissue extracts collected at post-mortem examination were prepared and analysed by Western blots. PGHS-2 was induced in ovine cotyledon in a time-dependent fashion following glucocorticoid injection ($P<0.05$). There was a 12-fold increase in abundance between the time of betamethasone administration (0 h) and established labour (56 h). The PGHS-2 isozyme was not detected in any of the other tissues examined. In contrast, formation of the PGHS-1 isozyme did not change in relation to induced-labour in any of the intrauterine tissues. This finding is consistent with constitutive formation of PGHS-1.

Previous studies have demonstrated a rise in PG production in association with glucocorticoid-induced labour and spontaneous delivery. The results of the present study indicate that this rise in PG production is due to increased formation of the PGHS-2 isozyme in ovine cotyledon. PGHS-2 appears to be induced by exogenous glucocorticoid administration and/or the mechanisms controlling ovine parturition. The role of PG formation by the fetal membranes is yet to be elucidated.

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Introduction

Parturition in the sheep is preceded by activation of the fetal hypothalamic–pituitary–adrenal axis (Liggins *et al.* 1973). Cortisol production from the fetal adrenal gland provides the trigger for labour onset by inducing changes in maternal progesterone concentrations, resulting in a

concomitant increase in placental oestrogen output (Flint *et al.* 1976). The rise in the oestrogen/progesterone ratio enhances the sensitivity of the myometrium to stimulatory agonists such as prostaglandin (PG) $F_{2\alpha}$ (PGF_{2 α}), PG E₂ (PGE₂) and oxytocin (Liggins *et al.* 1973). Furthermore, the change in the steroid milieu increases the secretion rate of PGF_{2 α} from uterine tissues, including the maternal

placenta, endometrium and fetal membranes (Challis *et al.* 1972, Currie *et al.* 1973, Taylor *et al.* 1982).

Dexamethasone, a glucocorticoid receptor agonist, has been shown to stimulate PGE₂ formation by human amnion cells in culture (Mitchell *et al.* 1988, Potestio *et al.* 1988). Similarly, PGF_{2α} production increases several-fold in maternal placental cotyledons and uterine venous plasma following labour induction in the ewe (Liggins & Grieves 1971). Evidence suggests that the effects of glucocorticoids on PG production are mediated by an increase in the protein content (Boshier *et al.* 1991, Rice *et al.* 1992) or activity (Rice *et al.* 1988, 1990) of PG G/H synthase (PGHS). The ability of the anti-progesterone and anti-glucocorticoid, RU486, to block the stimulatory effect of dexamethasone on PGE₂ output by human amnion cells suggests that the effect is receptor-mediated (Potestio *et al.* 1988).

Two PGHS isozymes have been identified and sequenced. PGHS-1, initially cloned and purified from ram seminal vesicular glands (Merlie *et al.* 1988), is a homodimer with a subunit molecular mass of 70 kDa. PGHS-1 protein formation is constitutive and it is thought to play an important role in cellular housekeeping functions (Wang *et al.* 1993). PGHS-2 conversely, shares approximately 60% amino acid sequence homology with PGHS-1 (Xie *et al.* 1991). Unlike PGHS-1, PGHS-2 content is transiently induced by a variety of agonists, including growth factors, phorbol esters and bacterial exotoxin (Kujubu *et al.* 1991, O'Sullivan *et al.* 1992, Hamasaki *et al.* 1993).

The rise in the PG synthetic capacity of intrauterine tissues brought about by glucocorticoid-induced parturition serves as an important model to determine whether corticosteroids are temporally related to the induction of PGHS. We have previously characterised and validated such an experimental model (McLaren *et al.* 1996). Intrafetal injection of the glucocorticoid betamethasone (5.7 mg) induced labour within 57 h in the ewe and resulted in endocrine profiles analogous to those observed at spontaneous-onset delivery. In the present study, we have utilised this model to characterise the localisation and developmental changes in PGHS-1 and PGHS-2 in ovine intrauterine tissues in the period immediately preceding labour onset. The results obtained have been compared with those of intrauterine tissues collected from ewes at spontaneous parturition.

Materials and Methods

Spontaneous labour animals

Six Border-Leicester/Merino cross-bred ewes of known gestational age were allowed to progress to spontaneous parturition. Ewes underwent surgery between 117 and 125 days of gestation to suture electromyographic (EMG) electrodes onto the surface of the myometrium. At day 140

of gestation, the EMG electrodes were connected to a polygraph recorder to monitor alterations in uterine activity. When ewes had been exhibiting signs of increased myometrial contractile activity characteristic of labour (Harding *et al.* 1982) for a period of 6–8 h, the ewe and fetus were killed by an overdose of barbiturate. Subsequently, a post-mortem examination was performed to collect tissues for Western blot analyses.

Labour induction and experimental protocol

Twenty Border-Leicester/Merino cross-bred ewes were used for labour induction. Labour was induced in ewes by the intrafetal administration of glucocorticoid on day 131 of gestation. Injection procedures were performed with the ewe held in a reclined or sitting attitude to minimise stress. Food was withheld from the ewe 8–12 h before ultrasonic examination to reduce intestinal gas.

The anterior abdominal wall of the sheep was closely shaved with fine animal clippers and sterilised with 70% ethanol. The fetus was then imaged with an ultrasound system (Diagnostic Ultrasound Equipment, model SDL-30; Shimadzu Corporation, Kyoto, Japan). The transducer was placed on the shaved area of skin (or on the bare inguinal region adjacent to the udder) to project an image on the monitor. The fetal heart and chest were visualised to ascertain the spatial orientation of the fetus. The fetal lamb was then injected i.m. in the shoulder or the hindlimb using a sterile 9 cm 20-gauge spinal needle (Terumo Medical Corporation, Tokyo, Japan).

Animals were administered 5.7 mg betamethasone (Celestone Chronodose in 1 ml aqueous vehicle; Schering Plough Pharmaceuticals, Baulkham Hills, NSW, Australia) using ultrasound guidance on day 131 of gestation (time 0). Sterile Indian ink was mixed with the glucocorticoid prior to injection. The position of the injection was then verified at post-mortem examination by the localisation of Indian ink staining. To examine the temporal pattern of PGHS-1 and PGHS-2 protein formation following glucocorticoid injection, ewes were killed at 0, 14, 28, 42 or 56 h post-injection ($n=4$). At the time of killing, ewes in the 56 h time group had been exhibiting signs of labour consistently over 6–8 h. Labour onset was determined from clinical factors such as swelling of the maternal vulva, and in some cases discharge of amniotic fluid. Animals were killed by the i.v. administration of a lethal dose (20 ml) of 325 mg/ml sodium pentobarbitone (Lethobarb; Arnolds of Reading Pty, Ltd, Peakhurst, NSW, Australia). A post-mortem examination was conducted and tissues were collected for Western blot analysis.

Tissue collection

Tissues collected at post-mortem examination, including cotyledons, amnion, chorion, allantois, myometrium, endometrium and cervix were excised in the shortest

possible time to prevent tissue necrosis and proteolytic degradation. Sampling was restricted to the midline of the ventral uterus, midway between the cervix and the bifurcation of the uterine horns. Endometrial tissue samples were taken from inter-caruncular regions. Tissues were immediately placed in liquid nitrogen before storage at -70°C .

Solubilisation of tissue extracts

Microsomal cell extracts were prepared as previously described (McLaren *et al.* 1996). Approximately 1.5 g wet weight of tissue were thawed to 4°C and homogenised. Prior to preparation, tissues were cleaned of connective tissue and washed free of clots in ice-cold Tris-buffered saline (TBS; pH 7.5) containing 20 mM Tris, 0.5 M NaCl, 0.1 M polymethylsulphonyl fluoride and 5 mM EDTA. Cotyledonary tissue samples had tissue of the zona intima dissected free from the capsule and central haemophagous segment prior to homogenisation. Tissue was homogenised for two 20 s bursts using a metal blade homogeniser (Ultra Turrax T25; IKA-Labortechnik, Staufen, Germany). Homogenisation of tissue was performed on ice in TBS (pH 7.5) at 5:1 w/v. The homogenate was then centrifuged at 10 000 *g* for 10 min at 4°C (Sorvall centrifuge; Newtown, CT, USA). The supernatant was collected and the pellet discarded. The cell extract was then centrifuged at 148 000 *g* for 60 min at 4°C . The resulting microsomal preparation was solubilised in TBS (pH 7.5) containing 0.1% Tween-20 (TTBS) and stored at -70°C until electrophoretic analysis.

Protein content determination

The protein concentration of solubilised microsomal cell extracts was determined using the method of Bradford (1976). BSA (CSL Ltd, Parkville, Victoria, Australia) was used to make reference standards ranging in concentration from 0 to 20 μg . Bio-Rad (Hercules, CA, USA) dye reagent concentrate was used for colour development. The depth of colour, measured at an absorbance wavelength of 720 nm (Titretek Multiskan MC, type 340; Eflab Oy, Helsinki, Finland), was analysed after 10 min against a reagent blank prepared from distilled water. The amount of protein was plotted against the corresponding absorbance, resulting in a standard curve used to determine the protein in unknown samples.

Generation of the PGHS-1 antibody

A polyclonal PGHS-1 antiserum was raised in female New Zealand white rabbits (1.5 kg body weight; $n=3$). PGHS used for injection (from ram seminal vesicle, 99% purity) was purchased from Oxford Biochemicals Company (Oxford, MI, USA). Animals were immunised by multiple-site s.c. injection of PGHS (60 μg in 300 μl

saline:Freund's complete adjuvant (1:1 v/v) per rabbit), three times at intervals of 2 weeks. Booster immunisations were administered, thereafter, at intervals of 6 weeks. Six months after the initial immunisation, rabbits were bled and serum was collected. The antiserum was then stored at -20°C until required for use. The cross-reaction of the antibody with the PGHS-2 isozyme was estimated to be $<0.1\%$, as determined from laser densitometry (McLaren *et al.* 1996).

Generation of the PGHS-2 antibody

A polyclonal sheep antiserum, specific against the PGHS-2 isozyme, was raised by immunisation with a peptide synthesised by Chiron Mimotopes Pty, Ltd (Clayton, Victoria, Australia). An 18 amino acid PGHS-2 C-terminal peptide (Ac-SRSGGLDDINPTVLLKERS-NH₂) was synthesised and covalently coupled to diphtheria toxoid via a cysteine residue at the N-terminal end of the peptide sequence (97% purity). The peptide-carrier conjugate was injected into two sheep, at 100 nmol peptide per injection, on day 1 using Freund's complete adjuvant and on day 14 using Freund's incomplete adjuvant. Prior to immunisation with the peptide, the sheep were bled to obtain a pre-immune serum sample. Both sheep were then bled on day 35 (3 weeks after the second injection) to obtain hyperimmune anti-peptide serum samples. Because the antiserum was raised against an 18 amino acid sequence present in the C-terminal portion of the PGHS-2 isozyme (O'Banion *et al.* 1992), and the sequence is not present in the PGHS-1 molecule, the antiserum did not cross-react with PGHS-1 isozyme.

SDS-PAGE

Microsomal cell extracts (50–150 μg protein/lane; refer to figure legends), PGHS-1 or PGHS-2 standards and high molecular mass markers were prepared for electrophoretic separation as previously described (McLaren *et al.* 1996). Samples were heated to 100°C for 2 min in SDS-sample loading buffer (99 mM Tris, 2.0% SDS, 2 mM EDTA, 10% glycerol and 0.05% bromophenol blue, pH 9.2; 30 μl /sample). Following denaturation, 2 μl 2-mercaptoethanol were added to each sample. Samples were then separated on 6–15% polyacrylamide gradient gels using an SE 600 Dual Cooled Vertical Slab Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, USA). A current of 10 mA and an offset voltage of 340 V was applied to the gel for approximately 16 h for electrophoretic separation of proteins. Proteins were then transferred to nitrocellulose membranes (0.45 μm) using a 2117–250 NovaBlot Electrophoretic Transfer kit (LKB Produkter AB, Bromma, Sweden). A current of 0.8 mA/cm² of membrane (100 V offset voltage) was applied for a period of 2 h to achieve complete transfer.

Non-specific binding was blocked by incubating the membrane with TTBS for 30 min. PGHS-1 antibody was used at a final dilution of 1:250 in TTBS diluent. PGHS-2 antibody was used at a final dilution of 1:12 000 in TTBS diluent. Membranes were incubated with the primary antibody for 1 h. To enable detection of the primary PGHS-2 antibody using ^{125}I -Protein A, membranes incubated with the Chiron PGHS-2 antiserum (raised in sheep) were subsequently incubated with rabbit anti-sheep immunoglobulins at a final dilution of 1:8000 for 30 min. The coefficient of variation (CV) of replicate samples of the same tissue within a single Western blot analysis using the PGHS-1 antiserum was 11.02%. The CV of replicate samples between analyses was 13.64%. The CV of replicate samples of the same tissue within a single Western blot analysis using the Chiron PGHS-2 antiserum was 10.67%. The CV of replicate samples between analyses was 13.12%.

^{125}I -Protein A, supplied in 0.05 M sodium phosphate buffer (pH 4.0) containing 35% ethanol (DuPont NEN Products, Boston, MA, USA), was used to detect binding of the primary antibody to the nitrocellulose membrane. Protein A (a component of the cell wall of *Staphylococcus aureus*) binds to the Fc portion of most immunoglobulin classes; however, it is non-reactive with antibodies derived from the sheep. Binding with rabbit immunoglobulins is strong. Iodinated tracer (approximately 1×10^6 c.p.m. in 30 ml TTBS) was incubated with Amberlite resin IRA-400 (BDH Chemicals, Kilsyth, Victoria, Australia) prior to incubation with the membrane to remove any free (unbound) ^{125}I . Membranes were incubated with the radiolabel for 2 h. Membranes were then washed in TTBS three times (20 min washes). At the conclusion of the washing step, membranes were monitored with a hand-held Mini-monitor (Series 900; Neomedix Systems, Sydney, Australia) to ensure that no radioactivity was present in regions of the filter that carried no protein.

Membranes were exposed to X-ray film (Kodak XAR-5; Eastman Kodak Company, Rochester, NY, USA) for 12–48 h with an intensifying screen. Autoradiographs were analysed by laser densitometry (Molecular Dynamics, Sunnyvale, CA, USA) and image analysis software (Image Quant, Molecular Dynamics). The amount of PGHS-1 or PGHS-2 protein loaded in the standard lanes was plotted against the corresponding density of the signal, resulting in a standard curve used to determine the enzyme content in unknown samples. The relationship between the amount of standard loaded on the gel and the densitometric value obtained was linear for both isoforms. Results for unknown samples are expressed as nanograms PGHS-1 or PGHS-2 per microgram of protein loaded.

Statistical analyses

Statistical analysis of the data was performed using a commercially available statistics package (Statistical

Packages for Social Sciences (SPSS-X)) Information Analysis System; SPSS Inc., Chicago, IL, USA). Data were first analysed by univariate homogeneity of variance tests (Bartlett Box F and Cochran's C test). If the raw data were inhomogeneous, the tests were repeated using $\log_{10}$ transformation and square root transformation. The transform most closely attaining homogeneity was then used for all subsequent statistical analyses. Western blot data were analysed by ANOVA, where time and tissue type were the factors. The post-hoc test of least significant difference 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 their untransformed state. Values are expressed as mean $\pm$ S.E.M.

Results

Spontaneous parturition

PGHS-1 immunoreactivity Immunoreactive PGHS-1 in ovine intrauterine tissues at term labour is shown in Fig. 1. PGHS-1 appeared as a 70 kDa band. In ovine cotyledon, a smaller 66 kDa fragment was also detected. The smaller immunoreactive protein has been reported previously in rats and sheep (Sirois & Richards 1992, Wimsatt *et al.* 1993, McLaren *et al.* 1996) and is believed to correspond to a proteolytic breakdown product. Alternatively, it may represent an N-glycosylation variant of the PGHS-1 enzyme (Otto *et al.* 1993). The fetal membranes (amnion, chorion and allantois) and maternal uterine tissues (myometrium, endometrium and cervix) did not demonstrate immunoreactivity at the 66 kDa level. The reason for this occurrence is not known.

PGHS-1 was weakly expressed in ovine placenta at term labour (Fig. 1A), reaching a mean concentration of 0.22 ± 0.04 ng/ μg protein loaded onto the gel (Fig. 2). The migratory pattern of the cotyledonary tissue extracts on the Western blot was slightly slower than that of the purified PGHS-1 standard. The reason for this occurrence, however, is not known. In contrast to sheep placenta, PGHS-1 was strongly expressed in amnion (Fig. 1B) and cervix (Fig. 1D) at spontaneous parturition. Densitometric quantitation of the bands demonstrated mean concentrations of 4.22 ± 0.72 and 3.01 ± 0.43 ng/ μg protein loaded respectively (Fig. 2). PGHS-1 concentrations in chorion and allantois were also high; however, final levels achieved were significantly lower than those observed in amnion (1.05 ± 0.36 and 1.59 ± 0.08 ng/ μg protein loaded respectively (Fig. 2). PGHS-1 was weakly expressed in endometrium and myometrium. The mean concentration of PGHS-1 in inter-caruncular endometrial samples at term labour was 0.39 ± 0.14 ng/ μg protein loaded. Myometrial samples reached a mean concentration of 0.55 ± 0.06 ng/ μg protein loaded (Fig. 2).

Figure 1 Western blot analysis of PGHS-1 in cotyledon and allantois (A), amnion and chorion (B), myometrium and endometrium (C) and cervix (D) at spontaneous labour. The first three lanes of each blot contain decreasing concentrations of purified PGHS-1 standard. The following lanes of each blot were loaded with solubilised microsomal cell extracts prepared from the respective tissues. In ovine placenta, PGHS-1 appeared as a 70 kDa protein and a smaller 66 kDa fragment. One hundred and fifty micrograms of protein were loaded into each lane for the placental, myometrial, endometrial and cervical tissue extracts. Fifty micrograms of protein were loaded into each lane for the amniotic, chorionic and allantoic membranes.

PGHS-2 immunoreactivity PGHS-2 in cotyledonary tissue extracts collected at term labour is shown in Fig. 3A. The PGHS-2 isozyme (estimated to be a 70 kDa fragment) was strongly expressed in ovine placenta at term labour. Formation of PGHS-2 in ovine cotyledon was

significantly greater ($P < 0.001$) than that observed for PGHS-1. The mean concentration of PGHS-2 at term labour was 19.18 ± 2.23 ng/ μ g protein loaded (Fig. 3B). In contrast, PGHS-2 was not detected in the fetal membranes (amnion, chorion and allantois) and maternal

Figure 2 The mean ($\pm$ S.E.M.) concentration of PGHS-1 in various intrauterine tissues ($n=6$, except where shown) at spontaneous labour, as determined from laser densitometry of Western blot autoradiographs. Data groups denoted by different letters represent significant differences between means ($P<0.05$). Groups sharing a letter are not significantly different ($P>0.05$). The mean gestational age of the animals at the time of parturition was 149 ± 0.5 days (mean $\pm$ S.E.M.).

gestational tissues (myometrium, endometrium and cervix). These tissues did not express an immunoreactive band that co-migrates with PGHS-2 standard (data not shown).

Glucocorticoid-induced labour

PGHS-1 and PGHS-2 protein content in ovine cotyledon The temporal patterns of PGHS-1 and PGHS-2 in ovine cotyledon relative to glucocorticoid-induced labour are shown in Fig. 4A and B respectively. PGHS-1 was constitutively expressed in sheep placenta prior to glucocorticoid injection. Tissue sections collected on day 131 of gestation (0 h; time of injection of betamethasone) demonstrated weak immunoreactive bands on the Western blot autoradiograph. Quantitation of PGHS-1 formation by ovine cotyledon demonstrated a 3.5-fold increase in protein levels between the time of glucocorticoid administration (0 h) and induced-labour (56 h; $P<0.05$; Fig. 5A).

In contrast, immunoblots probed with the PGHS-2 antibody demonstrated a large increase in protein content (Fig. 4B). There was a 12-fold increase in protein levels between the time of glucocorticoid administration and

Figure 3 (A) Western blot analysis of PGHS-2 in cotyledon at spontaneous parturition. The first three lanes of the Western blot contain decreasing concentrations of purified PGHS-2 standard. The following six lanes of the blot were loaded with solubilised cotyledonary tissue extracts. One hundred and fifty micrograms of protein were loaded into each lane. (B) The mean $\pm$ S.E.M. concentration of PGHS-2 in various intrauterine tissues ($n=6$) at spontaneous parturition, as determined from laser densitometry of Western blot autoradiographs. N/D=not detected.

induced-labour (Fig. 5B). Enzyme induction had occurred as early as 14 h post-injection, with maximal levels observed at labour onset (56 h post-injection). Interestingly, PGHS-2 protein levels were 22-fold higher than those observed for PGHS-1 in ovine cotyledon at the time of intrafetal glucocorticoid administration (0 h). The differential pattern of formation of the isozymes of PGHS in ovine placental tissue substantiates our previous data (McLaren *et al.* 1996) and working hypothesis that PGHS-2 is the principal enzyme induced in preparation for labour.

PGHS-1 and PGHS-2 protein content in ovine fetal membranes

The regulation of PGHS enzymes in the

Figure 4 Western blot analysis of PGHS-1 (A) and PGHS-2 (B) in cotyledonary tissue extracts following intrafetal glucocorticoid administration. The first three lanes of each blot contain decreasing concentrations of purified standard. The next ten lanes were loaded with solubilised microsomal extracts prepared from sheep cotyledons collected at the time of injection glucocorticoid (0 h) or at 14, 28, 42 or 56 h post-injection. One hundred and fifty micrograms of protein were loaded into each sample lane.

fetal membranes was markedly different from that detected in cotyledon. PGHS-1 protein levels were high in amnion (Fig. 6A), chorion (Fig. 6B) and allantois (Fig. 6C) when compared with cotyledonary samples. The pattern of PGHS-1 formation recognised by the antibody was similar in all the fetal membranes, with a transient fall in signal intensity observed 28 h after injection with glucocorticoid. The reason for this fall in protein levels is not known. Quantitative comparisons of PGHS-1 formation by ovine amnion with the other fetal membranes demonstrated higher immunoreactivity at all the time points examined. At the time of injection (0 h), PGHS-1 in amnion was 24.5 times higher than that observed in chorion and 6.5 times higher than that observed in allantois (Fig. 7A–C). PGHS-1 in chorion and allantois was significantly increased 14 h after intrafetal injection with glucocorticoid ($P < 0.05$); however, enzyme formation was not induced in response to labour onset.

When duplicate blots were probed for PGHS-2, no immunoreactivity was detected (data not shown). PGHS-2 was not present in the fetal membranes at the time of injection of betamethasone (0 h). Moreover, PGHS-2 enzyme formation was not induced in response to induced-labour.

PGHS-1 and PGHS-2 protein content in maternal intrauterine tissues Enzyme production in the myometrium, endometrium and cervix was markedly different from that observed in the fetal membranes. PGHS-1 immunoreactivity was minimal and did not change in response to induced-labour (data not shown). There was no consistent pattern of PGHS-1 protein levels across all the time points examined. Densitometric analysis of the Western blot autoradiographs revealed that mean PGHS-1 protein levels in myometrial, endometrial and cervical tissue extracts were significantly lower ($P < 0.05$) than

Figure 5 Mean ($\pm$ S.E.M.) concentrations of PGHS-1 (A) and PGHS-2 (B) (as determined from laser densitometry of Western blot autoradiographs) in placental tissue extracts obtained at various time intervals after intrafetal injection with betamethasone ($n=4$ at each time point). Data groups denoted by different letters represent significant differences between means ($P<0.05$). Groups sharing a letter are not significantly different ($P>0.05$).

those in the fetal membranes (data not shown). As was demonstrated with the fetal membranes, PGHS-2 protein was not found in maternal uterine tissues. Moreover, enzyme production was not induced by the mechanisms of labour.

Discussion

Normal parturition in the sheep is associated with a marked increase in concentrations of $\text{PGF}_{2\alpha}$ in utero–

ovarian vein plasma (Mitchell *et al.* 1979) and uterine tissues (Risbridger *et al.* 1985). The concentration of PGE_2 in fetal plasma increases before delivery (Challis *et al.* 1976). Similarly, the concentration of PGE_2 and $\text{PGF}_{2\alpha}$ in amniotic fluid is elevated in late pregnancy (Mitchell *et al.* 1977). Enhanced PG production by intrauterine tissues is a prerequisite for the increased myometrial contractile activity observed at term delivery.

Previously, it was thought that the release of arachidonic acid (AA) (the substrate for PG synthesis) from intracellular stores was the rate-limiting step in the formation of PGs (Flower 1980). Evidence now suggests that enhanced PGHS synthesis and activity rather than phospholipase activity is rate limiting for prostanoid formation. In sheep, the capacity of dispersed trophoblast cells to synthesise prostanoids is low up to 100–110 days of gestation. During this period, prostanoid synthesis cannot be stimulated by exogenous AA ($40\text{ }\mu\text{M}$) (Risbridger *et al.* 1985). This suggests that the activity and/or tissue content of PGHS is low. From 110 days of gestation, PG synthesis increases until term. The rise in PG formation is consistent with an increase in the rate of synthesis of PGHS and/or an increase in the half-life of the enzyme.

The aim of the present study was to examine the temporal pattern of PGHS-1 and PGHS-2 enzyme content in ovine intrauterine tissues using a glucocorticoid-induced labour model and to compare the results with those observed at term delivery. We have previously characterised and validated such a model (McLaren *et al.* 1996). The period from the point of intrafetal injection of betamethasone (5.7 mg) to killing following labour onset was $56.6 \pm 0.8\text{ h}$ (mean $\pm$ S.E.M.). Major endocrine changes recognised in the ewe and fetus before parturition at term were shown to occur before induced premature delivery. Maternal progesterone concentrations decreased significantly ($P<0.05$). This alteration in steroid biosynthesis is consistent with the induction of 17α -hydroxylase and C_{17-20} lyase activities, precipitating the formation of androgens from progesterone or pregnenolone (Flint *et al.* 1976). In addition, PG concentrations in fetal, maternal and utero–ovarian vein plasma were elevated.

In sheep, cotyledonary PGHS activity and immunoreactive content rises in the last third of gestation, with a rapid increase associated with labour onset (Mitchell *et al.* 1979). Fowden *et al.* (1987) demonstrated enhanced placental PGE_2 secretion into the maternal and fetal circulations in late gestation. Evans *et al.* (1981) showed that following adrenocorticotrophic hormone-induced labour, PGF and 6-keto $\text{PGF}_{1\alpha}$ levels were significantly increased in cotyledons and chorioallantois. Rice *et al.* (1988) demonstrated that the capacity of microsomes prepared from ovine cotyledons to synthesise PGE_2 and $\text{PGF}_{2\alpha}$ from radiolabelled AA increased 25-fold between 20 and 140 days of gestation. Our results support the hypothesis that PGHS-2 is the enzyme mostly responsible for increased PG formation by ovine placenta both at term

Figure 6 Western blot analysis of PGHS-1 in amnion (A), chorion (B) and allantoic (C) membranes. The first three or four lanes of each blot contain decreasing concentrations of purified PGHS-1 standard. The next ten lanes were loaded with solubilised microsomal cell extracts prepared from the respective fetal membranes collected at the time of injection of glucocorticoid (0 h) or at 14, 28, 42 or 56 h post-injection. Fifty micrograms of protein were loaded into each lane.

delivery and induced-labour onset. The transient increase in PGHS-2 following glucocorticoid administration and subsequent labour onset suggests that this isozyme is regulated by the mechanisms controlling parturition in the ewe.

PGHS-2 in sheep placenta, as detected by Western blot analysis, demonstrated a 12-fold increase between glucocorticoid injection and ensuing labour onset (see Fig. 5B).

A 3-fold increase in protein levels was detected as early as 14 h post-injection. Furthermore, PGHS-2 protein was present in placental extracts on day 131 of gestation (0 h). The presence of the PGHS-2 isozyme prior to day 131 of gestation parallels that of fetal adrenal gland activation. Endogenous cortisol concentrations begin to rise approximately 25 days before delivery (Magyar *et al.* 1980). Similarly, PGHS activity begins to increase at

Figure 7 Mean $\pm$ S.E.M. concentration of PGHS-1 (as determined from laser densitometry of Western blot autoradiographs) obtained from solubilised fetal amnion (A), chorion (B) and allantoic (C) membrane extracts probed for PGHS-1 protein by Western blot analysis. Tissues were obtained from four animals at each time point, unless otherwise specified. Points sharing a letter are not significantly different at $P=0.05$.

110–125 days of gestation in the ewe (Rice *et al.* 1988), with a rapid increase in the last 2 weeks of pregnancy.

The temporal pattern of PGHS-2 induction demonstrated by this study is consistent with the endocrine data measured previously to validate the experimental model of intrafetal glucocorticoid administration (McLaren *et al.*

1996). Glucocorticoid-induced labour was shown to elicit a significant increase ($P<0.05$) in arterial plasma concentrations of PGE_2 , $\text{PGF}_{2\alpha}$ and PGFM (the inactive metabolite of $\text{PGF}_{2\alpha}$) in the circulation of fetal lambs. In addition, a rise in the concentrations of $\text{PGF}_{2\alpha}$ and PGFM in maternal arterial and uterine venous plasma was observed in response to induced delivery. It was concluded that the increase in prostanoid formation was mediated by the induction of placental PGHS-2 enzyme content. The results from the present study strongly support this hypothesis. Since PGHS is characterised by a short biological half-life (<10 min), the increase in PGHS-2 protein levels shown by the present investigation is consistent with a sustained rise in the rate of synthesis of the enzyme.

In contrast to the PGHS-2 isozyme, PGHS-1 protein levels in ovine cotyledon were low at the time of spontaneous parturition. Furthermore, PGHS-1 protein content increased only slightly following betamethasone administration. This small increase, however, may have been due to partial cross-reaction of the PGHS-1 antibody with the PGHS-2 isozyme. Previous investigations have demonstrated no change in the level of PGHS-1 mRNA (Rice *et al.* 1995, Gibb *et al.* 1996) or protein content (Wimsatt *et al.* 1993) in association with labour onset in the sheep.

The ovine fetal membranes represent a major source of PG output in late gestation. Olson *et al.* (1986) measured concentrations of PGE_2 , $\text{PGF}_{2\alpha}$, PGFM, 6-keto $\text{PGF}_{1\alpha}$ and 6-keto PGE_1 on day 131 of pregnancy. PGE_2 concentrations were high in chorioallantois and amnion tissue extracts. Moreover, Evans *et al.* (1982) measured intra-uterine tissue concentrations of PGE_2 , $\text{PGF}_{2\alpha}$ and 6-keto $\text{PGF}_{1\alpha}$ at different stages of pregnancy in sheep. The concentrations of these PGs in chorioallantois and amnion were significantly higher on days 130 and 145 than on days 50 or 100 of pregnancy.

The results of the present study suggest that PGHS-1 is the enzyme responsible for prostanoid production by ovine fetal membranes. PGHS-1 protein content was high in amnion, chorion and allantois when compared with cotyledonary tissue at term delivery and induced-labour onset. Conversely, the PGHS-2 isozyme was not synthesised in the fetal membranes and was not induced by glucocorticoid administration and subsequent labour onset. Protein levels of PGHS-1 in amnion were generally 2–3 times higher than those expressed by the other fetal membranes. This finding is consistent with the high output of PGs by dispersed amnion cells *in vitro* (Evans *et al.* 1982). This result also supports the findings of Langlois *et al.* (1993), who demonstrated that the conversion of AA into PGs by amnion was significantly greater than by chorion between days 78 and 140 of gestation.

In contrast to the fetal membranes, PGHS-1 protein content in myometrial, endometrial and cervical tissue extracts was minimal at term delivery and in the time preceding induced-labour. Protein levels were not

consistent across the time points examined and did not increase with labour onset. Furthermore, PGHS-2 was not detected in maternal gestational tissues and did not increase in response to betamethasone-induced labour. These results are in direct contrast to the findings of Zhang *et al.* (1996), who demonstrated elevated levels of PGHS-2 in myometrium and endometrium during glucocorticoid-induced premature labour. The reason for this discrepancy in results is not known and can only be resolved through further experimentation.

In conclusion, this study has delineated the localisation and pattern of PGHS-1 and PGHS-2 enzyme content in ovine intrauterine tissues in relation to the initiation of parturition in the ewe. Increased PGHS-2 protein content in sheep placental tissue appears to be the main factor responsible for the pre-partum increase in prostanoid formation observed at term delivery. PGHS-1 protein in the fetal membranes does not change in response to labour while PGHS-1 protein content in maternal gestational tissues is minimal. The exact role of PG production by these tissues is yet to be elucidated.

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