Expression of prostanoid receptor genes in baboon chorion and decidua during pregnancy and parturition

G C S Smith, W X Wu and P W Nathanielsz

Laboratory for Pregnancy and Newborn Research, Department of Physiology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, USA

(Requests for offprints should be addressed to G C S Smith, University of Glasgow, Department of Obstetrics and Gynaecology, The Queen Mother's Hospital, Yorkhill, Glasgow G3 8SH, UK; Email: gcs4@cornell.edu)

Abstract

The aim of this study was to determine whether expression of prostanoid receptor genes varied with gestational age or labour in the decidua and chorion of baboons. Tissues were obtained at the time of Caesarean hysterectomy in 15 baboons, 10 prior to the onset of labour in the last third of pregnancy and 5 in spontaneous labour at term. Expression of prostanoid receptor genes was determined using Northern blot analysis and the level of expression was related to each of three housekeeping genes. Expression of the genes encoding the EP₁ and FP receptor in decidua and the EP₄ receptor in chorion was

lower with advancing gestational age. Expression of the EP₂ receptor gene was lower in labour in decidua, whereas expression of the IP receptor gene was higher in labour in both decidua (twofold) and chorion (fourfold). It is concluded that there is a complex pattern of change in expression of prostanoid receptor genes in chorion and decidua with advancing gestational age and in association with labour. It seems likely that direct effects of prostaglandins on the choriodecidua may have an important role in parturition in the primate.

Journal of Endocrinology (2001) **168**, 263–272

Introduction

The fetal membranes and decidua are thought to have a key role in the control of parturition in the human and non-human primate. In analyses of cases of preterm labour, approximately 30–40% of births are associated with prelabour rupture of the membranes (Creasy & Iams 1999). Furthermore, the membranes have an important paracrine role in the control of myometrial contractility, both in spontaneous term labour (Challis *et al.* 1997) and in preterm labour secondary to intra-uterine infection (French & McGregor 1996). The decidua is also thought to have an important paracrine role in the control of the myometrium (Casey & MacDonald 1988).

Currently, most models for the role of prostaglandins (PGs) in the fetal membranes and decidua consider the membranes as a source of either PG biosynthesis or a site of PG catabolism (Challis *et al.* 1997). Furthermore, any effects of PGs from the fetal membranes and decidua are thought to be paracrine, mediated through changes in myometrial contraction (French & McGregor 1996). The genes encoding a family of at least eight distinct prostanoid receptors have been cloned and sequenced and match closely with pharmacologically defined receptor types and sub-types (Coleman *et al.* 1994). We recently demonstrated a complex pattern of expression of these genes in the chorion and decidua of

pregnant baboons (Smith *et al.* 1998), which suggests that PGs synthesised in the membranes and decidua may also have autocrine effects.

We hypothesise that altered sensitivity to prostaglandins mediated by variation in the population of prostanoid receptors expressed in critical intra-uterine tissues may have a role in the control of parturition in primates. Characteristically, genes encoding signal transduction proteins which have a role in labour are expressed at higher levels in tissues (both myometrial and non-myometrial) from animals which are in labour, and there are many examples of such phenomena (Tabb & Garfield 1992, Wu *et al.* 1996, Ou *et al.* 1997, Slater *et al.* 1999, Wu *et al.* 1999). As an initial test of our hypothesis that PG receptors have a direct role in the control of the chorion and decidua around labour, we sought to determine whether expression of these genes varied according to gestational age and whether the animal was in labour or not in labour at the time the tissue was obtained.

Materials and Methods

Care and use of animals

Animal care and surgical procedures have been described in detail previously (Morgan *et al.* 1992). Pregnant

baboons were obtained from the Southwest Foundation for Biomedical Research, San Antonio, TX, USA. They had been harem mated and gestational age was confirmed by early ultrasound. Total Caesarean hysterectomy was performed under general anaesthesia (ketamine induction, halothane maintenance).

At the time of surgery, 10 animals were not in labour and were in the last third of pregnancy at the following days gestational age (dGA): 121, 128, 141, 153, 159, 162, 162, 177, 177, 180 (term=180–185 days). The cervix was un-effaced and closed in all of these animals. Uterine electromyogram leads had been sited in three of these animals going close to term (delivered at 177, 177 and 180 dGA). Analysis of electromyogram (EMG) traces of the 48 h preceding surgery revealed no contraction activity. No drugs of any form had been administered to any of the animals in the two weeks preceding surgery. Hysterectomies prior to the onset of labour were performed over a period of two years. In some analyses, samples were not available for all animals from this pool. Caesarean hysterectomy was also performed on five animals in spontaneous labour. Of these, four had EMG electrodes sited. Labour was diagnosed by cervical dilatation and effacement. These animals had a baseline cervical examination at the time of their first EMG contraction activity and a repeat cervical examination was performed when they had a sustained switch from contractures to contractions (>30 min). Hysterectomy was performed on the basis of cervical changes. The gestational ages at hysterectomy were 164, 184, 191, 193 days. The cervical dilations for these animals were 6 cm, 3 cm, 3 cm and 2 cm respectively (the cervix was closed in all four at the baseline examination). In a fifth animal without EMG electrodes, Caesarean section was performed at 172 dGA and at the time of the Caesarean section it was found that the cervix was 4 cm dilated and fully effaced and a hysterectomy was performed.

The chorion and amnion were separated and flash frozen in liquid nitrogen. Decidua were removed following separation of the fetal membranes and were flash frozen. All procedures were approved by the Cornell University Institutional Animal Care and Use Committee and the facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

Northern analysis

Polyadenylated ribonucleic acid (RNA) was extracted from frozen tissue by oligo-thymidylic acid-cellulose affinity chromatography using a commercial kit (Fast Track 2.0, Invitrogen, San Diego, CA, USA). Samples of polyadenylated RNA (2 or 4 µg) were denatured in 17.4% (vol/vol) formaldehyde, 50% (vol/vol) freshly deionised formamide, 20 mM MOPS (3-(N-morpholino) propanesulfonic acid), 5 mM sodium acetate and 1 mM

EDTA, pH 7.0, for 5 min at 65 °C and separated by electrophoresis on a 1.4% (wt/vol) agarose-0.66 M formaldehyde gel. The gel was visualised under ultraviolet trans-illumination and photographed to determine the distance of migration of a series of standard size RNA markers (Gibco, MD, USA). The RNA was transferred onto a nylon membrane (Gene Screen plus, NEN Dupont, MA, USA) by capillary blotting for 24 h in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). Pre-hybridisation (>1 h) and hybridisation (>18 h) were carried out in hybridisation bottles in an oven at 65 °C when using riboprobes and at 45 °C in sealed bags when a cDNA probe was used. A commercial 50% formamide based hybridisation buffer was employed (Northern Max or UltraHyb, Ambion, Austin, TX, USA). The probe concentration was approximately 1×10^6 counts per min per ml hybridisation buffer. Membranes were washed twice for five min in 2 × SSC and 0.1% SDS at 65 °C and twice for one hour in 0.1 × SSC and 0.1% SDS at 65 °C when riboprobes were employed and were washed in the same buffers but at 45 °C when cDNA probes were employed. Kodak X-Omat film was exposed to the membrane with an intensifying screen at -80 °C. After probing for the receptor gene of interest, membranes were stripped (see below) and re-probed for housekeeping genes (see below).

Synthesis of probes

The EP₁, EP₂, EP₃, EP₄, FP, IP and DP prostanoid receptor complementary deoxyribonucleic acids (cDNAs) had been cloned into either the pcDNA3 or pcDNAIamp vectors (both Invitrogen), which include promoters for phage polymerases SP-6 and T-7. The plasmid was linearized by an appropriate restriction enzyme and antisense riboprobes were synthesised using a commercial kit (StripEZ RNA, Ambion) labelled with [α -³²P]UTP (800 Ci/mmol) (NEN Dupont). Template DNA was removed by addition of 2 U RNase-free DNase and incubation for 15 min at 37 °C. cDNA probes were used where riboprobes could not be synthesised and the inserts were labelled with [α -³²P]deoxy-CTP (3000 Ci/mmol) using the random priming method (StripEZ DNA, Ambion) to specific activities of approximately 1×10^9 c.p.m./µg. Both DNA and RNA probes was separated from unincorporated nucleotide using a Sephadex spin column (Probe Quant G-50, Pharmacia Amersham, NJ, USA) and quantified. Probes were stripped from membranes using the manufacturer's protocol (Strip EZ RNA and Strip EZ DNA, both Ambion) and membranes were consecutively stripped and re-probed to each of three housekeeping genes.

The human EP₂ receptor cDNA was obtained from Dr D F Woodward of Allergen, Irvine, CA, USA. The human prostanoid receptor DP, EP₃, EP₄, IP, and FP cDNAs were obtained from Dr M Abramovitz of Merck

Figure 1 Northern blot of mRNA from decidua (either 2 or 4 μ g each lane, same quantity loaded in each lane in a given blot) probed for (A) EP₁ receptor gene, (B) EP₂ receptor gene, (C) EP₃ receptor gene and (D) EP₄ receptor gene. All membranes were consecutively stripped and re-probed for housekeeping genes. The estimated size of transcripts is given in kilobases (kB). A: lanes 1–10 not in labour, lanes 11–15 in labour; B, C and D: lanes 1–9 not in labour, lanes 10–14 in labour.

Figure 2 Northern blot of mRNA from decidua (either 2 or 4 µg each lane, same quantity loaded in each lane in a given blot) probed for (A) FP receptor gene, (B) IP receptor gene and (C) TP receptor gene. All membranes were consecutively stripped and re-probed for housekeeping genes. The estimated size of transcripts is given in kilobases (kB). A and C: lanes 1–8 not in labour, lanes 10–14 in labour; B: lanes 1–9 not in labour, lanes 10–14 in labour. Lane 9 was empty in A and C.

Frosst, Quebec, Canada. The human TP receptor cDNA was obtained from Oxford Biomedical (MI, USA) and the mouse EP₁ receptor cDNA was obtained from Dr Y Sugimoto (Kyoto University, Japan). The plasmids (all

TRIsScript, Ambion) containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin and cyclophilin cDNAs with RNA polymerase promoters were purchased from Ambion.

Figure 3 Northern blot of mRNA from chorion (either 2 or 4 µg each lane, same quantity loaded in each lane in a given blot) probed for (A) EP₁ receptor gene, (B) EP₂ receptor gene, (C) EP₃ receptor gene and (D) EP₄ receptor gene. All membranes were consecutively stripped and re-probed for housekeeping genes. The estimated size of transcripts is given in kilobases (kB). A, B and D: lanes 1–8 not in labour, lanes 9–13 in labour; C: lanes 1–7 not in labour, lanes 9–13 in labour. Lane 8 was empty in C.

Statistical analysis

The estimated size of transcripts was calculated by fitting a curve (using Graph Pad Prism, version 3.0, Graph Pad Software, San Diego, CA, USA) to the graph of distance

of migration plotted against molecular weight of the markers. The size of autoradiographic signals was quantified using densitometry. All bands which were clearly greater than background were analysed densitometrically. The expression of a given prostanoid receptor gene was

Figure 4 Northern blot of mRNA from chorion (either 2 or 4 μ g each lane, same quantity loaded in each lane in a given blot) probed for (A) IP receptor gene and (B) TP receptor gene. All membranes were consecutively stripped and re-probed for housekeeping genes. The estimated size of transcripts is given in kilobases (kB). Both blots: lanes 1–7 not in labour, lanes 9–13 in labour. Lane 8 was empty in both plots.

expressed as a ratio to each of three housekeeping genes: GAPDH, beta-actin and cyclophilin. Comparison of two means was made by Student's *t*-test. Correlation was determined using simple linear regression and Spearman's rho. Comparison of means adjusted for gestational age was

performed using analysis of covariance and the comparison normalised to 180 dGA. Statistical analysis was performed using Stata College Station, TX, USA, version 6.0 for Windows. Statistical significance was assumed at the 5% level.

Results

Clear signals of similar molecular weight to the cloned human prostanoid receptors genes were detected in decidua to probes for the EP₁, EP₂, EP₃, EP₄, FP, and IP receptors (Figs 1 and 2). No clear signal above background could be detected using an antisense riboprobe for the human DP receptor. Clear signals of similar molecular weight to the cloned human prostanoid receptors genes were detected in chorion to probes for the EP₁, EP₂, EP₃, EP₄, and IP receptors (Figs 3 and 4). No clear signal above background could be detected using antisense riboprobes for the human DP or FP receptor genes. Weak signals could be detected in both tissues to a TP receptor probe (Figs 2 and 4). However, the signal to noise ratio for the TP receptor gene transcript was too low in both tissues for meaningful quantitative analysis.

When related to the signal for beta-actin, there was a statistically significant negative correlation between the level of expression of the EP₁ and FP receptor genes in decidua and EP₄ expression in the chorion and the gestational age at the time of hysterectomy among animals which were not in labour (Fig. 5). There were no other significant relationships between gene expression and gestational age in either tissue (all $P > 0.05$). Results of similar statistical significance were obtained when correlation was tested using Spearman's rho (data not shown).

Signals obtained from animals in labour and not in labour were related to beta-actin and compared. There was a significantly lower level of expression of the EP₂ receptor gene in decidua among animals in labour (Fig. 6), but there was no significant difference comparing decidua from animals not in labour and those in labour for expression (all mean in arbitrary units (S.E.M.)) of the EP₁ receptor gene (0.47 (0.04) vs 0.37 (0.06) respectively, $P = 0.17$), the EP₃ receptor gene (7.7 kB transcript = 1.38 (0.09) vs 1.41 (0.11) respectively, $P = 0.84$; 5.5 kB transcript = 1.66 (0.11) vs 1.85 (0.14) respectively, $P = 0.32$; 2.6 kB transcript = 1.25 (0.09) vs 1.36 (0.07) respectively, $P = 0.42$) and the EP₄ receptor gene (0.98 (0.09) vs 1.18 (0.23) respectively, $P = 0.35$). There was no significant difference comparing chorion from animals not in labour and those in labour for expression (all mean in arbitrary units (S.E.M.)) of the EP₁ receptor gene (0.85 (0.08) vs 0.89 (0.15) respectively, $P = 0.79$), the EP₂ receptor gene (0.42 (0.09) vs 0.43 (0.08) respectively, $P = 0.91$), the EP₃ receptor gene (7.7 kB transcript = 0.48 (0.08) vs 0.36 (0.04) respectively, $P = 0.24$; 5.5 kB transcript = 0.47

Figure 5 Simple linear regression of prostanoid receptor gene expression (expressed as a ratio to beta-actin) against gestational age among animals not in labour. (A) EP₁ receptor gene in decidua ($r^2=0.7$, $P=0.002$, $n=10$), (B) FP receptor gene in decidua ($r^2=0.7$, $P=0.008$, $n=8$), (C) EP₄ receptor gene in chorion ($r^2=0.7$, $P=0.01$, $n=8$). Signals were quantified using densitometry and are expressed as a ratio (arbitrary units) to signal for beta-actin.

(0.07) vs 0.45 (0.04) respectively, $P=0.78$; 2.6 kB transcript = 2.21 (0.28) vs 1.45 (0.13) respectively, $P=0.06$, the EP₄ receptor gene (1.55 (0.29) vs 0.95 (0.14) respectively, $P=0.16$) or the FP receptor gene (0.47 (0.10) vs 0.26 (0.09) respectively, $P=0.19$). There was, however, a significantly higher level of IP receptor gene expression in both decidua and chorion obtained from animals in labour (Fig. 7).

For the signals which were observed to vary with gestational age (EP₁ and FP in decidua and EP₄ in chorion) a further comparison was made of tissues obtained from animals in labour and animals not in labour, where the comparison was adjusted for gestational age using analysis of covariance. In all three

cases there was still no significant change in levels of expression associated with labour after adjusting for the effect of gestational age (EP₁ decidua, $P=0.65$; FP decidua, $P=0.95$; EP₄ chorion, $P=0.65$).

All the above comparisons were made expressing the level of the given gene as a ratio to beta-actin. Significant results were also observed when levels were expressed relative to one of the other housekeeping genes, with one exception. There was a negative correlation of borderline statistical significance between gestational age and EP₁ expression in chorion relative to beta-actin ($P=0.05$). This association was not statistically significant when the EP₁ signal was related either to GAPDH ($P=0.87$) or to cyclophilin ($P=0.11$).

Figure 6 Densitometric analysis of expression of prostanoid EP₂ receptor gene expression (expressed as a ratio to beta-actin) in decidua comparing labour (LAB, $n=5$) and not in labour (NIL, $n=9$). Comparison of LAB and NIL by Student's *t*-test. Columns are means and bars are S.E.M.

Discussion

Four patterns of change in expression of prostanoid receptor genes in the decidua and chorion were observed in this study. The first pattern observed was a decrease in expression of genes encoding receptors which are positively coupled to the phosphatidyl inositol cascade (EP₁ and FP) in decidua with advancing gestational age (Fig. 5A and B). The parallel decrease in these receptors with advancing gestational age suggests that a prostanoid-mediated response acting through the phosphatidyl inositol cascade might inhibit changes in the decidua which promote labour. However, EP₁ and FP receptor activation stimulate myometrial contractility (Senior *et al.* 1993). Therefore, these data suggest that EP₁ and FP receptor-mediated effects may stimulate parturition-promoting processes in myometrium and may inhibit parturition-promoting changes in others.

The second pattern that emerged was a decrease in the expression of a gene which encodes a receptor (EP₄) which is positively coupled to adenylate cyclase in chorion with advancing gestational age (Fig. 5C). This suggests that an adenylate cyclase-mediated effect may inhibit changes in chorion which promote parturition. This parallels myometrium, where adenylate cyclase-coupled receptors inhibit myometrial contractility (Senior *et al.* 1993).

The third pattern was a lower level of expression of the gene encoding an adenylate cyclase-coupled receptor (EP₂) in decidua obtained from animals in spontaneous labour (Fig. 6). This suggests that an adenylate cyclase-mediated effect may inhibit parturition-related processes in decidua in cells expressing the EP₂ receptor. This parallels decreased EP₂ receptor expression in myometrium and cervix (Smith *et al.* 1999). We hypothesise that reduced expression of this critical receptor may have a key role in promoting parturition in the primate. The absence of any change in expression of the EP₂ receptor

A. Decidua

B. Chorion

Figure 7 Densitometric analysis of expression of prostanoid receptor IP gene (expressed as a ratio to beta-actin) in labour (LAB, $n=5$) and not in labour (NIL, $n=7-9$) for (A) decidua and (B) chorion. Comparison of LAB and NIL by Student's *t*-test. Columns are means and bars are S.E.M.

in chorion suggests differential transcriptional control in maternal and fetal intra-uterine tissues.

The fourth pattern, observed in both the decidua and chorion, was an increase in the expression of a gene encoding an adenylate cyclase-coupled receptor (IP) in labour and this was particularly marked in the chorion (Fig. 7A and B). This increased expression suggests that adenylate cyclase activation might promote parturition in cells expressing the IP receptor in these tissues. It was interesting to observe that two adenylate cyclase-coupled prostanoid receptors (EP₂ and IP) vary in opposite directions in the same tissue in association with labour. This underlines the potential for the same second messenger system to mediate different effects in different cells types

and this presumably has its basis in different effects of protein kinase A in different cells. Activation of the IP receptor in myometrium inhibits myometrial contractility (Senior *et al.* 1993) and the observed increase in IP receptor gene expression, like decreased EP₁ and FP expression in decidua with advancing gestational age, suggests dissociation of the roles of receptors in choriodecidua compared with other key tissues. The present findings suggest that extreme caution should be applied in any studies seeking to exploit the IP receptor pathway as a means of uterine tocolysis, since any inhibitory, therapeutic effect on the myometrium may be undermined by effects on the choriodecidua.

The data presented in this study are confined to analysis of mRNA. Ideally, we would have confirmed that message was translated into protein using Western blot analysis and that this is reflected in the presence of receptors using radio-ligand binding. However, antibodies against the currently recognised prostanoid receptors are not available and radio-ligand binding is limited due to the lack of truly specific ligands (Coleman *et al.* 1990). However, previous studies of key systems involved in parturition have initially focused on mRNA and highlighted physiologically important regulators of the process, such as nitric oxide (Ali *et al.* 1997) and cyclo-oxygenase-2 (COX-2) (Slater *et al.* 1999). While our studies on prostanoid receptors in these tissues are, therefore, preliminary, we hypothesize that the observed changes in mRNA may reflect an important role for a direct effect of PGs on the control of the choriodecidua in labour. The pattern sizes of bands observed in the present study in the baboon were very close to those previously described in Northern analysis of human prostanoid receptor gene expression (Hirata *et al.* 1991, An *et al.* 1993, Funk *et al.* 1993, Boie *et al.* 1994, Lake *et al.* 1994, Regan *et al.* 1994, Kotani *et al.* 1995).

There are relatively few data on prostanoid receptors in the fetal membranes and decidua. A previous study from this laboratory failed to demonstrate variation in either EP₂ or EP₄ receptor gene expression in labour in ovine endometrium in association with labour (Ma *et al.* 1999). However, species differences between the sheep and baboon have previously been demonstrated for EP₂ receptor-mediated effects on myometrium (Senior *et al.* 1993, Crankshaw & Gaspar 1995, Garcia-Villar *et al.* 1995). A number of studies have postulated a role for prostanoid receptors in the endometrium in association with implantation (Yang *et al.* 1997). An important role for PGs in this context is supported by the failure of implantation and decidualisation in mice which are null mutant for the COX-2 gene (Lim *et al.* 1997), and this may be due to the absence of PGI₂ (Lim *et al.* 1999). Other studies have demonstrated expression of the EP₁ receptor in amnion WISH cells. Expression of the EP₁ receptor in these tissues is stimulated by corticotrophin-releasing hormone (CRH) (Spaziani *et al.* 2000), interleukin-1β

(Spaziani *et al.* 1999), interleukin-4 (Spaziani *et al.* 1997) and tumour necrosis factor α (Spaziani *et al.* 1998). Interestingly, the effect of CRH appeared to be mediated by PGE₂ (Spaziani *et al.* 2000), suggesting the possibility of a feedback loop. However, the data presented in this study are the first, to our knowledge, to demonstrate a changing pattern of prostanoid receptor gene expression with advancing gestational age and in labour.

Acknowledgements

Financial support was supplied by NIH (HD 21350). G C S S was supported by the Wellcome Trust.

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Received 12 October 2000

Accepted 18 October 2000