Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy

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ABSTRACT

Human labour is associated with increased prostaglandin synthesis within the fetal membranes. We have studied the expression of the two isoforms of the central prostaglandin synthetic enzyme, cyclo-oxygenase (COX-1 and COX-2), in human fetal membranes throughout pregnancy, at mRNA, protein and activity levels.

COX-1 mRNA expression was low in human amnion and chorion–decidua and did not change with gestational age. COX-2 mRNA expression in fetal membranes increased with gestational age, with significant up-regulation prior to the onset of labour and in association with labour. Protein concentrations of COX-1 did not change, whilst concentrations of COX-2 increased from the first to the third trimester. COX activity increased with gestational age and in association with labour, although prostaglandin production in fetal membranes collected after labour was reduced, suggesting reduced substrate supply.

These data suggest that it is up-regulation of COX-2, rather than of COX-1, which mediates increased prostaglandin synthesis within the fetal membranes at term. Much of the increase in COX-2 expression precedes the onset of labour, suggesting that it is a cause, rather than a consequence, of labour.

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INTRODUCTION

There is considerable evidence to support a central role for prostaglandins in human parturition. Labour is associated with increased prostaglandin synthesis within the uterus (Keirse *et al*. 1977), particularly from the fetal membranes (Skinner & Challis 1985). Prostaglandins mediate cervical ripening, and directly stimulate uterine contractions (Crankshaw & Dyal 1994) and indirectly increase fundally dominant myometrial contractility by up-regulation of oxytocin receptors and synchronisation of contractions (Liggins 1989, Garfield *et al*. 1990).

Prostaglandins are formed from the precursor arachidonic acid, which itself is a substrate for at least three enzyme groups. The cyclo-oxygenase (COX) pathway produces prostaglandins. The lipoxygenase enzyme pathways produce a series of hydroxyeicosatetraenoic acids (HETEs). Arachidonic acid metabolism via epoxygenase pathways produces epoxyeicosatetraenoic acids. Prior to labour, endogenous arachidonic acid metabolism in amnion is principally via the lipoxygenase enzyme pathways. With labour, there is an increase in arachidonic acid metabolism and a change in the ratio of COX to lipoxygenase metabolism to favour synthesis of prostaglandin E_2 (Saeed & Mitchell 1982, Bennett *et al*. 1993). The roles of the lipoxygenase metabolites of arachidonic acid within the uterus are unknown, although 5-HETE may play a role in pre-labour (Braxton–Hicks) contractions (Bennett *et al*. 1987, Walsh 1989).

The switch from lipoxygenase to COX metabolism implies up-regulation of COX. Two COX genes have been described, the constitutively expressed COX-1 and the inducible COX-2 (Hla *et al*. 1986, Hla & Neilson 1992). *In situ* hybridisation has localised expression of COX-1 and COX-2 mRNA in the fetal membranes (Slater *et al*. 1995). Expression of COX-2 is mainly within the amnionic epithelial layer and also in cells of the amnionic mesoderm, and in the reticular layer of both chorion and decidua. COX-2 is not expressed in the trophoblast layer of the chorion. COX-1 expression is more diffuse, and is seen in the amnionic epithelium, amnionic mesoderm and also in the chorionic mesoderm and decidua. In amnion

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at term, expression of the COX-2 mRNA is some 100-fold higher than that of COX-1 mRNA. The expression of COX-2 doubles in association with labour (Slater *et al*. 1994, 1995, Hirst *et al*. 1995). In chorion–decidua at term, COX-2 mRNA is 8-fold more abundant than COX-1 mRNA, and COX-2 expression increases 4-fold in association with labour (Slater *et al*. 1998). Enzyme kinetic studies suggest that the increase in COX activity with the onset of labour is due entirely to an increase in the synthesis of the COX enzyme (Smieja *et al*. 1993).

There is conflicting evidence as to whether the increased prostaglandin synthesis associated with labour is a cause or an effect of labour. MacDonald & Casey (1993) found that the increase in prostaglandin concentrations in the amniotic fluid occurred earlier and was greater in the fore-bag (in front of the fetal head) compared with the hind-bag, and that hind-bag prostaglandin concentrations in early labour were not increased above pre-labour concentrations. They inferred from these data that prostaglandin formation was a consequence of labour. However, studies where sequential amniocentesis was performed, on patients before and during labour, showed that increased prostaglandin concentrations preceded the onset of labour (Romero *et al*. 1996).

In this study we have examined COX-1 and COX-2 mRNA, protein and activity levels in human fetal membranes at different stages of pregnancy. We have tested the hypothesis that up-regulation of COX-2 within the human fetal membranes occurs before the onset of labour and is therefore a cause, and not a consequence, of labour.

MATERIALS AND METHODS

Tissue samples

For mRNA studies, fetal membranes were collected with consent and ethics committee approval between 6 and 22 weeks at surgical termination of pregnancy, between 28 and 37 weeks at elective caesarean section prior to the onset of labour, in cases of maternal cardiac, renal or hypertensive disease, and after 38 weeks at elective caesarean section prior to labour in cases of breech presentation or previous caesarean section.

Reverse transcription–polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Slater *et al*. 1995). Primers used were: COX-2 sense (5′-TTCAAATGAGATTTGGGAAAATTGCT), COX-2 antisense (5′-AGATCATCTCTGCCTGAGTATCTT), COX-1 sense (5′-TGCCCAGCTCCTGGCCCGCCGCTT), COX-1 antisense (5′-CCATGGCCCAAGGCCTTG), glyceraldehyde 3-phosphate dehydrogenase (GAP) DH sense (5′-CCACCCATGGCAAATTCCATGGCA), GAPDH antisense (5′-TCTAGACGGCAGGTCAGGTCCACC) (Hla *et al*. 1986, Hla & Neilson 1992). The reaction cycles were denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s.

To determine the exponential phase of amplification, where product formation is related to starting template concentration, a cycle profile was performed for each tissue. An average sample was created by mixing equal amounts of cDNA from all of the RT reactions from a single tissue type. PCR was performed using the 'average sample' template cDNA for between 20 and 40 cycles using primers specific for COX-1, COX-2 and GAPDH. PCR products were dotted onto Hybond N membranes (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) and hybridised overnight to the appropriately radiolabelled cDNA probe (Slater *et al*. 1994). Membranes were washed to high stringency conditions with 0·1 × sodium citrate buffer at 65 °C, and Cerenkov counted (Johnson 1969). In these experiments optimal cycle numbers were: 35 for COX-1 in amnion and chorion–decidua, 31 and 33 for COX-2 in amnion and chorion–decidua respectively, and 22 for GAPDH in all three tissues. These cycle numbers were used in subsequent experiments. To control for RNA concentration the expression of COX-1 and COX-2 was calculated as a ratio to that of GAPDH for each individual sample.

Western analysis

Protein extracts were prepared from fetal membrane samples by homogenisation in 10 vols T-Wash (50 mM Tris buffer, 10 mM EDTA, 1% Triton-100, with 10 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin and 0·5 μg/ml leupeptin) for 30 s. Proteins (20 μg) were separated on a 10% SDS-PAGE gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked in 5% Marvel, 0·1% Tween in PBS and incubated with either COX-1 or COX-2 specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500. Membranes were washed in 0·1% Tween in PBS and incubated with anti-goat IgG–horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2000. Antibody specificity was confirmed using positive controls. Human platelets were used as a

positive control for COX-1 and interleukin-1β-stimulated human lymphocytes for COX-2. Western autoradiographs were quantified by digital densitometry.

Fetal membrane COX activity and prostaglandin synthesis

Prostaglandin synthesis from exogenous substrate sources by fetal membranes was measured to assess COX activity. Membranes were washed in PBS, and 2 cm discs of separated amnion and chorion were cut and suspended in serum-free medium 199 for 1 h. Membrane discs were then incubated with fresh medium containing arachidonic acid (30 μM) for 15 min. Prostaglandin E_2 concentration in the medium was measured by RIA according to manufacturer's instructions (Sigma-Aldrich, Poole, Dorset, UK). To assess prostaglandin synthesis from endogenous substrate sources intact fetal membrane discs were suspended in 1·5 ml serum-free medium 199 with insulin (1 μM), transferrin (0·1 μM) and selenium (0·05 μM) (Sigma-Aldrich) for 2 h. Prostaglandin E_2 concentration was measured by enzyme immunoassay (Amersham Pharmacia Biotech).

Statistical analysis

Data were analysed in groups, respectively estimating the mean and standard error before comparison was made using one way analysis of variance (ANOVA). Differences between groups were assessed using Fisher's exact test. Statistical significance was assumed if $P<0·05$.

RESULTS

COX mRNA expression, as a ratio to the expression of the constitutively expressed GAPDH, was compared among ten gestational age groups. COX-1 mRNA expression in amnion and in chorion–decidua was low throughout pregnancy and did not change with gestational age or the onset of labour (Fig. 1). COX-2 mRNA expression in amnion and in chorion–decidua was low or undetectable until the second trimester, when it then increased towards term and doubled with labour (Fig. 2). ANOVA showed a significant effect of gestational age and labour on COX-2 expression both in amnion and in chorion. There was no correlation between COX-1 expression and gestational age.

Western analysis showed COX-2 protein at 72 kDa and COX-1 protein at 70 kDa. Protein

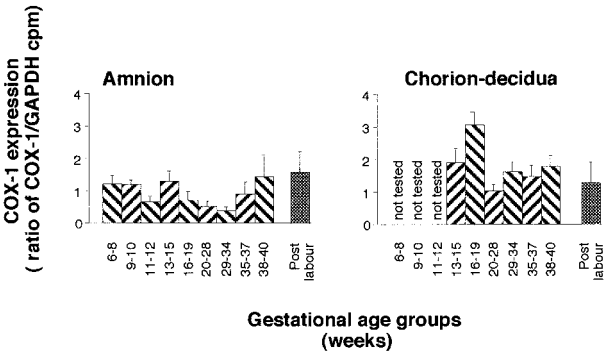

FIGURE 1. Expression of COX-1 mRNA, by RT-PCR, in amnion and chorion–decidua throughout gestation. Results are expressed as means and S.E. of the ratio of c.p.m. COX-1/GAPDH from gestational age groups; 6–8 weeks (n=4), 9–10 weeks (n=4), 11–12 weeks (n=6), 13–15 weeks (n=8), 16–19 weeks (n=4), 20–28 weeks (n=4), 29–34 weeks (n=6), 35–37 weeks (n=6), 38–40 weeks (prior to labour) (n=6), and 38–40 weeks (after labour) (n=6).

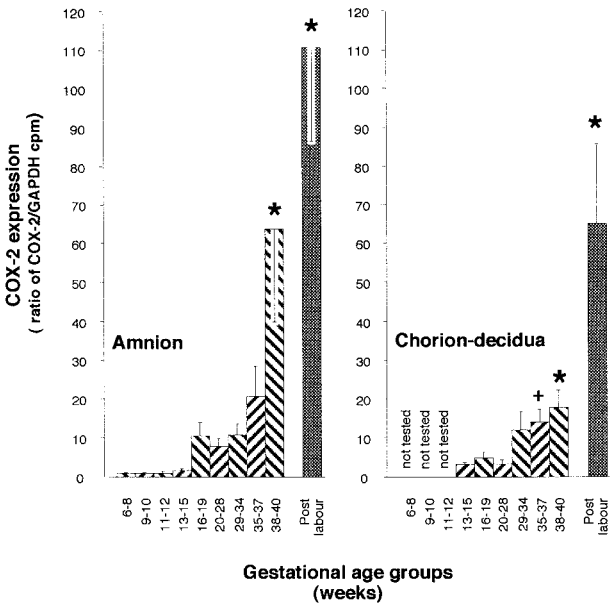

FIGURE 2. Expression of COX-2 mRNA, by RT-PCR, in amnion and chorion–decidua throughout gestation. Results are expressed as means and S.E. of the ratio of c.p.m. COX-2/GAPDH from gestational age groups; 6–8 weeks (n=4), 9–10 weeks (n=4), 11–12 weeks (n=6), 13–15 weeks (n=8), 16–19 weeks (n=4), 20–28 weeks (n=4), 29–34 weeks (n=6), 35–37 weeks (n=6), 38–40 weeks (prior to labour) (n=6), and 38–40 weeks (after labour) (n=6). In both amnion and chorion–decidua ANOVA showed a significant effect of gestational age upon COX-2 expression ($P<0·05$). *$P<0·05$ compared with all other groups; +$P<0·05$ compared with all groups below 28 weeks.

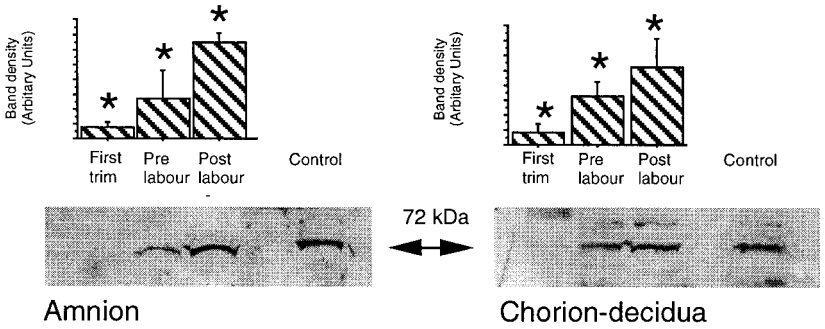

FIGURE 3. Western blot analysis of COX-2 protein in fetal membranes. Lower panels show original gels; upper panels show densitometric analysis of blots (means ± S.E., n=4). Expression of COX-2 protein was compared in fetal amnion and chorion–decidua from gestational age groups; 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38–40 weeks (after labour). *P<0·05 compared with all other groups. P values as assessed by ANOVA and Fisher's exact test.

concentrations of COX-1 were similar at all gestational ages and did not change significantly with gestational age or with labour in either amnion or chorion–decidua (data not shown). In amnion, protein concentrations of COX-2 increased from almost undetectable levels in the first trimester to maximum levels in samples collected after labour (Fig. 3). In chorion–decidua, COX-2 protein concentration increased significantly between the first trimester and term. There was also a significant difference between chorion–decidua collected before and after labour.

COX activity was assessed in three gestational age groups, 10–14 weeks, 38–40 weeks prior to labour, and 38–40 weeks after labour. In amnion, COX activity increased between the first and third trimesters and was further increased in tissues collected following labour (Fig. 4). In chorion–decidua there was no change in activity between the first and third trimesters, but there was a significant increase in activity following the onset of labour. Production of prostaglandin E_2 from endogenous substrate sources in intact fetal membranes increased between the first and third trimesters, but there was a decrease post-labour when compared with pre-labour (Fig. 5).

DISCUSSION

During pregnancy the uterus expands to accommodate the growing fetus and placenta whilst remaining relatively quiescent, and the cervix remains firm and closed. At term these changes reverse to allow the uterus to contract, and the cervix to soften and dilate. Lye (1994) has suggested that labour is the result of the activation of a 'cassette of contraction associated proteins' which might include gap junction proteins, oxytocin and prostaglandin receptors. We have previously shown that, at the mRNA level, it is principally COX-2 which is expressed in both amnion and chorion–decidua, and that its expression increases with the onset of labour, whilst the expression of COX-1 in both tissues is unchanged (Slater *et al*. 1995, 1998). We

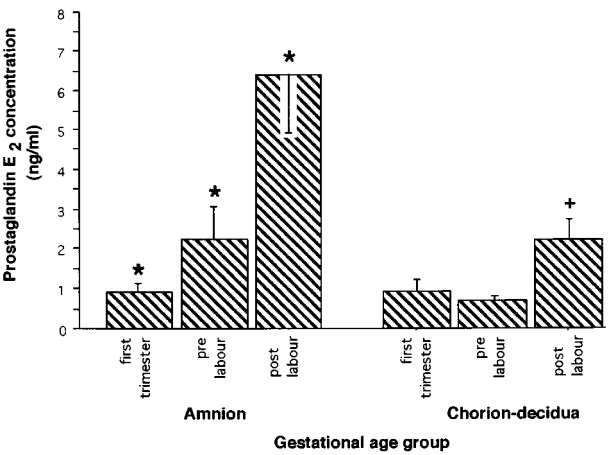

FIGURE 4. Assay of COX activity in amnion and chorion–decidua. Production of prostaglandin E_2 by fetal membranes from exogenous arachidonic acid was compared between 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38-40 weeks (after labour) (n=5). Membranes were incubated with 30 μM arachidonic acid for 15 min and prostaglandin E_2 production was measured by RIA. Means ± S.E., *P<0·05 compared with all other groups; +P<0·05 compared with term pre-labour group. P values as assessed by ANOVA and Fisher's exact test.

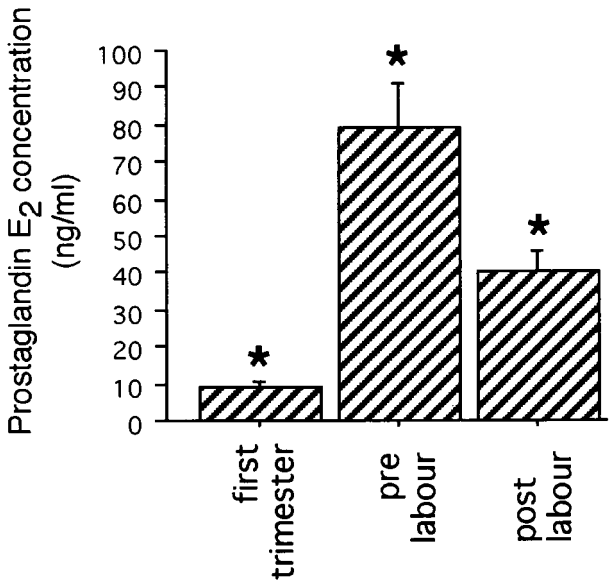

FIGURE 5. Production of prostaglandin E_2 by fetal membranes from endogenous arachidonic acid was compared between 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38–40 weeks (after labour) (n=5). Membranes were incubated in medium for 2 h and the prostaglandin E_2 produced measured by ELISA. Means ± S.E., *P<0·05 compared with all other groups. P values as assessed by ANOVA and Fisher's exact test.

have confirmed our hypothesis that the central prostaglandin synthetic enzyme COX might also be a member of the 'cassette of contraction associated proteins', by our finding of significant up-regulation of COX-2 expression in late pregnancy prior to labour. In sheep, the principal source of prostaglandins in late gestation is the placental cotyledon rather than the fetal membranes (Mitchell & Flint 1977, Rice *et al*. 1988). Up-regulation of COX-2 expression in sheep placenta takes place gradually, as pregnancy advances, and COX-1 expression does not change (Wimsatt *et al*. 1993, Rice *et al*. 1995). The temporal pattern of COX expression is therefore similar in the two species, but in different tissues.

The major differences in COX-2 mRNA expression occur between the first and third trimesters, and before and after labour. Because of the difficulty of obtaining adequate numbers of tissue samples in the second and early third trimester, we confined COX activity studies and Western analysis to tissues obtained in the first trimester and at term, before and after labour. Western analysis showed increases in COX-2 protein concentrations between the first and third trimester and before and after labour, paralleling observations at the mRNA level. We found no change in COX-1 expression at either the mRNA or protein level. These data, together with previous studies showing increased COX-2 expression in fetal membranes with the onset of labour (Hirst *et al*. 1995, Slater *et al*. 1995, 1998), suggest that it is up-regulation of COX-2 in fetal membranes which mediates the increased prostaglandin synthesis associated with labour and delivery in the human.

Prostaglandin synthesis requires liberation of substrate arachidonic acid through the activity of a phospholipase. We have studied COX activity by measuring synthesis of prostaglandins from exogenous arachidonic acid because both endogenous arachidonic acid concentrations and phospholipase A_2 activity are known to fall in fetal membranes during labour (Curbelo *et al*. 1981, Skannal *et al*. 1997). This assay eliminates constraints on prostaglandin synthesis by endogenous substrate depletion or reduced phospholipase activity, but it does depend upon prostaglandin synthase E activity to produce prostaglandin E_2. We also measured synthesis of prostaglandin E_2 from intact fetal membranes without exogenous arachidonic acid, to examine their overall capacity for prostaglandin synthesis. Prostaglandin synthesis from endogenous sources increased between the first and third trimesters, in parallel with the increase in COX-2 mRNA and protein concentrations. The decrease in prostaglandin synthesis in fetal membranes collected after labour is consistent with lower endogenous arachidonic acid concentrations (Curbelo *et al*. 1981) and phospholipase A_2 activity (Skannal *et al*. 1997) following labour. The combination of increased COX-2 mRNA and protein expression, and maximum prostaglandin synthesis from endogenous substrate sources prior to labour, shows that increased prostaglandin synthesis within the fetal membranes precedes, and is therefore a cause, rather than a consequence, of labour.

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REFERENCES

Bennett PR, Elder MG & Myatt L 1987 The effects of lipoxygenase metabolites of arachidonic acid on human myometrial contractility *in vitro*. *Prostaglandins* **33** 837–844.

Bennett PR, Slater DM, Sullivan MHF, Elder MG & Moore GE 1993 Changes in arachidonic acid metabolism in amnion cells associated with increased cyclo-oxygenase gene

expression at parturition. *British Journal of Obstetrics and Gynaecology* **100** 1037–1042.
Crankshaw DJ & Dyal R 1994 Effects of some naturally occurring prostanoids and some cyclo-oxygenase inhibitors on the contractility of the human lower uterine segment *in vitro*. *Canadian Journal of Physiology and Pharmacology* **72** 870–874.
Curbelo V, Bejar R & Benirschke K 1981 Prostaglandin precursors in human placental membranes. *Obstetrics and Gynaecology* **57** 473–478.
Garfield RE, Tabb T & Thilander G 1990 Intercellular coupling and modulation of uterine contractility. In *Uterine Contractility*, pp 21–40. Ed RE Garfield. Norwell, MA, USA: Serono Symposia.
Hirst JJ, Teixeira FJ, Zakar T & Olson DM 1995 Prostaglandin endoperoxide-H synthase-2 expression increases in human gestational tissues with spontaneous labour onset. *Reproduction, Fertility and Development* **7** 633–637.
Hla T & Neilson K 1992 Human cyclo-oxygenase-2 cDNA. *Proceedings of the National Academy of Sciences of the USA* **89** 7384–7388.
Hla T, Farrell M, Kumar A & Bailey JM 1986 Isolation of the cDNA for human prostaglandin H synthase. *Prostaglandins* **32** 829–845.
Johnson MK 1969 Counting of Cerenkov radiation from 32P in nonaqueous media. *Analytical Biochemistry* **29** 348–350.
Keirse MJ, Mitchell MD & Turnbull AC 1977 Changes in prostaglandin F and 13,14-dihydro-15-keto-prostaglandin F concentrations in amniotic fluid at the onset of and during labour. *British Journal of Obstetrics and Gynaecology* **84** 743–746.
Liggins GC 1989 Initiation of labour. *Biology of the Neonate* **55** 366–375.
Lye S 1994 The initiation and inhibition of labour – toward a molecular understanding. In *Seminars in Reproductive Endocrinology*, vol 12, pp 284–294. Ed MD Mitchell. New York, USA: Thieme Medical Publishers, Inc.
MacDonald PC & Casey ML 1993 The accumulation of prostaglandins (PG) in amniotic fluid is an after effect of labour and not indicative of a role for PGE_2 or $PGF_{2\alpha}$ in the initiation of human parturition. *Journal of Clinical Endocrinology and Metabolism* **76** 1332–1339.
Mitchell MD & Flint A 1977 Prostaglandin concentrations in intrauterine tissues from late pregnant sheep before and after labour. *Prostaglandins* **14** 563–569.
Rice GE, Wong MH & Thorburn GD 1988 Gestational changes in prostaglandin synthase activity of cotyledonary microsomes. *Journal of Endocrinology* **118** 265–270.
Rice GE, Freed KA, Aitken MA & Jacobs RA 1995 Gestational and labour associated changes in relative abundance of prostaglandin G/H synthase-1 and -2 mRNA in ovine placenta. *Journal of Molecular Endocrinology* **14** 237–244.
Romero R, Munoz H, Gomez R, Parra M, Polanco M, Valverde V, Hasbun J, Garrido J, Ghezz F, Mazor M, Tolosa JE & Mitchell MD 1996 Increase in prostaglandin bioavailability precedes the onset of human parturition. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **54** 187–191.
Saeed SA & Mitchell MD 1982 Formation of arachidonic acid metabolites by human fetal membranes, uterine decidua vera and placenta. *Prostaglandins, Leukotrienes and Medicine* **8** 635–640.
Skannal DG, Brockman DE, Eis AL, Xue S, Siddiqi TA & Myatt L 1997 Changes in activity of cytosolic phospholipase A_2 in human amnion at parturition. *American Journal of Obstetrics and Gynecology* **177** 179–184.
Skinner KA & Challis JRG 1985 Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor. *American Journal of Obstetrics and Gynecology* **151** 519–523.
Slater DM, Berger L, Newton R, Moore GE & Bennett PR 1994 The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term. *Biochemical and Biophysical Research Communications* **198** 304–308.
Slater DM, Berger L, Newton R, Moore GE & Bennett PR 1995 Changes in the expression of types 1 and 2 cyclo-oxygenase in human fetal membranes at term. *American Journal of Obstetrics and Gynecology* **172** 77–82.
Slater DM, Allport V & Bennett PR 1998 Changes in the expression of the type-2 but not the type-1 cyclo-oxygenase enzyme in chorion–decidua with the onset of labour. *British Journal of Obstetrics and Gynaecology* **105** 745–748.
Smieja Z, Zakar T, Walton JC & Olson DM 1993 Prostaglandin endoperoxide synthase kinetics in human amnion before and after labour at term and following preterm labour. *Placenta* **14** 163–175.
Walsh SW 1989 5-Hydroxyeicosatetraenoic acid, leukotriene C4 and prostaglandin $F_{2\alpha}$ in amniotic fluid before and during term and preterm labor. *American Journal of Obstetrics and Gynecology* **161** 1352–1360.
Wimsatt J, Nathanielsz PW & Sirois J 1993 Induction of prostaglandin endoperoxide synthase isoform-2 in ovine cotyledonary tissues during late gestation. *Endocrinology* **133** 1068–1073.

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