Labour is associated with increased expression of type-IIA secretory phospholipase A2 but not type-IV cytosolic phospholipase A2 in human myometrium

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Human labour is associated with increased prostaglandin synthesis within the uterus. The aim of this study was to examine the expression of the type-IV cytosolic phospholipase A2 (cPLA2-IV) and the type II A secretory phospholipase A2 (sPLA2-IIA) in myometrium in association with labour onset at term and preterm deliveries. These enzymes are important for the release of the prostaglandin precursor, arachidonic acid, from phospholipid membrane stores. RT-PCR was used to determine differences in gene expression between non-labour and labour groups. Expression of sPLA2-IIA in human myometrium was significantly increased with pregnancy, and with labour, both at term and preterm. Expression of cPLA2-IV in myometrium was not significantly altered with respect to pregnancy or labour. Immunohistochemical analysis demonstrated differences in the spatial localization of cPLA2-IV and sPLA2-IIA protein in upper and lower segment myometrium. cPLA2-IV was predominantly in vascular endothelial cells, while sPLA2-IIA was observed in vascular, endothelial and smooth muscle cells. In addition, sPLA2-IIA showed a distinct nuclear or perinuclear localization in myometrial smooth muscle cells of the lower segment. We postulate that the increased expression of sPLA2-IIA rather than cPLA2-IV in the myometrium may play a role in the onset and/or maintenance of human parturition.

Key words: cPLA2/labour/myometrium/preterm/sPLA2

Introduction

The molecular mechanisms for the onset and maintenance of labour at term are not fully understood; however, prostaglandins are strongly implicated (Skinner and Challis, 1985). The synthesis of prostaglandins can be divided into three main stages, each of which may be rate-limiting. The first involves the mobilization of the precursor arachidonic acid from membrane phospholipids, by phospholipase enzymes. The free arachidonic acid is then converted to the prostaglandin intermediate, prostaglandin H2 (PGH2) in the second step by the action of cyclo-oxygenase (COX). Finally PGH2 is converted to prostaglandins of the E, F, thromboxane, and prostacyclin series by specific PG synthase enzymes.

Two isoforms of COX, coded by two distinct genes, have been described; the constitutively expressed COX-1 and the inducible COX-2 (Hla et al., 1986; Hla and Neilson, 1992). COX-2, but not COX-1, expression increases in association with labour in fetal membranes and myometrium, and is thus likely to play a role in the increased prostaglandin synthesis observed within the uterus at term (Hirst et al., 1995; Slater et al., 1995, 1999; Smieja et al., 1993). Any increases in COX activity may also require a concomitant increase in the release of the prostaglandin precursor, arachidonic acid. This step is catalysed by the action of one or more phospholipase A2 (PLA2) enzymes, which release arachidonic acid from the sn-2 position of intracellular membrane phospholipids. Numerous distinct PLA2 isoforms have been identified, differing in molecular size, cellular localization, Ca2+ dependence and substrate specificity (Balsinde et al., 1999). They are broadly categorized into two classes; the secretory phospholipases (ranging from 13 to 18kDa) which require millimolar Ca2+ for catalysis, and the larger cytosolic forms (ranging from 30 to 110kDa) with no catalytic requirement for Ca2+. The secretory PLA2 (sPLA2) enzymes are further classified into groups I, II, III, V and X, and the cytosolic PLA2 (cPLA2) enzymes into groups IV, VI, VII and VIII (review: Six and Dennis, 2000; Gelb et al., 2000).

To date, only sPLA2 type-IIA, sPLA2 type-V and cPLA2 type-IV have been identified within the human uterus (Lappas and Rice, 2004). Human labour is not associated with changes in sPLA2-IIA expression or activity in human fetal membranes or placenta (Okazaki et al., 1981; Lopez Bernal et al., 1992; Aitken et al., 1990, 1996; Bennett et al., 1994; Freed et al., 1997). However, cPLA2-IV activity, in fetal amnion, is highest prior to labour and decreases following labour onset, suggesting that cPLA2-IV mobilization of arachidonic acid is highest immediately prior to and in anticipation of labour, but becomes depleted during labour (Skannal et al., 1997a). Both cPLA2-IV and sPLA2-IIA protein has been identified in lower segment pregnant human myometrium (Skannal et al., 1997b), but there is no available data on gestational or labour-associated changes.

The aims of this study therefore were to determine whether there were any changes in the mRNA expression of cPLA2 type-IV...
and or sPLA₂ type-IIA in human myometrium, in association with labour at term or preterm. To determine if there were any differences in the spatial localization of cPLA₂-IV or sPLA₂-IIA between upper and lower segment myometrial samples, we have performed immunohistochemical localization. In order to verify that mRNA expression levels correlate positively to those of the protein, we also analysed the relative abundance of cPLA₂-IV and/or sPLA₂-IIA mRNA and protein levels in fetal membranes and placenta by RT–PCR and western analysis respectively.

Materials and methods

Patient samples
All tissues were collected following informed written consent and institutional Ethics Committee approval (Walsgrave Hospital Trust, Coventry, UK). Myometrial samples were collected from the upper edge of the uterine incision during lower segment Caesarean section (lower segment samples) and paired samples were collected from the upper uterine segment with biopsy forceps (upper segment samples). Myometrial tissue was separated from any serosal or decidual components using a dissection microscope. Paired (upper and lower segment) myometrial biopsies were collected from women undergoing Caesarean section (CS) at term, not in labour (TNL) or in labour (TL), and preterm, not in labour (PTNL) or preterm in labour (PTL). The TNL group (n = 14) comprised a gestational age range of 37–40 weeks; reasons for CS were: maternal request, breech presentation or previous Caesarean section. The TL group (n = 9) comprised a gestational age range of 38–41 weeks and all with spontaneous onset of labour. All patients had regular contractions and cervical dilation (dilation ≥ 3 cm n = 2, 4–5 cm n = 2, 6 cm n = 1, 7 cm n = 1, 9 cm n = 2, full n = 1). Reasons for CS were: fetal distress (n = 5), previous section combined with high head and large baby (n = 1), interuterine growth retardation (IUGR) (n = 1), all without oxytocin or prostaglandin administration. Two patients had oxytocin administration and were delivered for failure to progress. The PTNL group (n = 7) had a gestational age range of 28–35 weeks. There was no evidence of uterine contractions or cervical change and reasons for CS were: fetal distress, twin pregnancy or IUGR. The preterm PTL group (n = 4) comprised a gestational age range of 27–35 weeks; reasons for CS were: fetal distress, transverse or breech presentation. Non-pregnant myometrium was obtained (n = 4) with consent from women undergoing hysterectomy for dysmenorrhoea. Fetal membranes and placenta were also collected at term at elective Caesarean section prior to labour (in cases of breech presentation of previous Caesarean section) or following spontaneous vaginal delivery. Tissues were rinsed in phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen and stored at −80°C prior to RNA or protein isolation. Paired upper and lower segment myometrium was also collected for immunohistochemical analysis. Myometrial samples were separated from serosal or decidual components, viewed under a dissection microscope.

RT–PCR
RNA was isolated using an SV Total RNA Isolation System (Promega, UK). RT–PCR was used for semi-quantitative analysis of RNA expression. Reverse transcription was carried out using random hexanucleotide primers, and the resultant complementary DNA (cDNA) used as template for PCR, using gene-specific primers. Total RNA (100–500 ng) was first denatured at 70°C for 5 min, followed by reverse transcription with Superscript II (Gibco BRL) at 37°C for 60 min. The resultant cDNA was utilized for subsequent PCR amplification as described previously (Slater et al., 1995). PCR primers (5′–3′) were: cPLA2-IV (GenBank accession number D38178) GAGCTGA-TGTTCGAGATTGGGTG (sense), GTCACTCAAGGAAGACATGGAA-TAAGA (antisense) (Clark et al., 1991; Sharp et al., 1991); sPLA2-IIA (accession number NM 000300) CCTGTGCTACTCATGACTGTT (sense), GGAGTACAGCTCCTTTGGA (antisense) (Selhamer et al., 1989); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CCACCATGGCC-AATTTCCATGGCA (sense), TCTAGACGCGACCTAGGCA (antisense). Product sizes were 509, 478 and 598 base pairs respectively. Cycling parameters were: denaturing, 94°C, 30 s; 55°C, annealing 30 s; extension, 72°C, 30 s for an appropriate number of cycles and followed by a 72°C, 5 min extension. Following amplification, PCR products were analysed by agarose gel electrophoresis, subcloned into the pGEM-8T Easy vector (Promega, UK) and verified by sequencing.

For each target gene to be analysed, a ‘cycle profile’ was performed to determine the linear range of amplification where product formation is related to starting template. An aliquot from each myometrial cDNA sample was taken and pooled. This ‘pooled’ sample was then used as template for PCR cycle profiles. Cycles were carried out from 20 to 36, at two cycle intervals. Following amplification, 10 μl aliquots of the reactions were subject to agarose gel electrophoresis and PCR products stained with ethidium bromide and visualized under UV light. Abundance of amplified product was performed by densitometric analysis of the gel using Total Lab software (Newcastle upon Tyne). Densitometric units were plotted against cycle number to establish the exponential phase of amplification. The appropriate cycle number within the linear range of amplification was chosen for subsequent analyses of each gene. The number of PCR cycles within the linear range of amplification was 30–36 and so 33 cycles were used for subsequent experiments to determine the relative abundance of either sPLA₂-IIA or cPLA₂-IV within the myometrium. Expression levels of cPLA₂-IV and sPLA₂-IIA mRNA were normalized to GAPDH.

Western blotting
Protein extracts were prepared from fetal membrane and placenta by homogenization in T-wash (50 mmol/l Tris buffer, 10 mmol/l EDTA pH 8.0, 1% Triton-100 with 10 mmol/l phenylmethylsulphonyl fluoride, 4 μg/ml pepstatin and 0.5 μg/ml leupeptin. Proteins (20 μg) were separated on a 10% sodium dodecyl sulphate–polyacrylamide gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Transfer efficiency and equal loading of proteins was assessed by staining with Poncette Red solution (Sigma). After transfer, membranes were washed with PBS-T (PBS, 0.1% Tween-20; Sigma) and following an incubation in blocking buffer, subsequently incubated overnight at 4°C, with cPLA₂-IV (Santa Cruz) (dilution 1/1000) or sPLA₂-IIA (Cayman Chemical) (dilution 1/1000) primary antibody. After incubation with the primary antibody, membranes were washed and incubated (1 h room temperature) with an Ig G horseradish peroxidase-conjugated secondary antibody (dilution1/10000). Thereafter membranes were washed in PBS-T and protein bands visualized by enhanced chemiluminescence western blotting detection (Amersham Pharmacia Biotech Ltd).

Immunohistochemistry
Myometrial tissue sections (5 mm) were deparaffinized in xylene and rehydrated by passing through a graded alcohol series. Antigen retrieval was performed using 1% antigen unmasking solution (Vector Laboratories) incubated at 96°C for 60 min. To localize the phospholipases the Vectastain Elite ABC detection kit (Vector) was used following the manufacturer’s protocol. Briefly, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide, tissue sections were then blocked in 10% antibody host serum in PBS at room temperature for 60 min and incubated overnight with the desired polyclonal primary antibody at 4°C. Primary antibodies, sPLA₂-IIA (Cayman Chemicals) and cPLA₂-IV (Santa Cruz) were diluted 1:100 in 10% serum/PBS. Incubation of the sections with pre-absorbed primary antibody gave no staining. The colour reaction was developed with a biotinylated secondary antibody (30 min room temperature), addition of avidin/biotinylated complex (30 min room temperature) followed by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution with metal enhancer (Sigma). Sections were counterstained using Harris haematoxylin (Sigma), dehydrated in an increasing ethanol series, cleared in xylene and the coverslips mounted in DPX mounting media (BDH).

Statistics
Data were analysed within groups, estimating the mean and SE. Statistical significance was determined using analysis of variance, followed by a post-hoc test with the StatView 4.5 statistics software (Abacus Concepts Inc., Berkeley, USA).
Results

cPLA2-IV mRNA was expressed in all pregnant and non-pregnant myometrial samples, while sPLA2-IIA was only expressed in pregnant myometrium (Figure 1).

There were no significant changes in the expression of cPLA2-IV mRNA with gestational age or labour in either upper or lower segment samples (Figure 2). By contrast, in lower segment myometrium, expression of sPLA2-IIA was significantly increased in association with labour both at term and preterm (Figure 3a). Expression of sPLA2-IIA was significantly higher in the PTL compared to PTNL (P < 0.05) and in the TL group compared to the TNL group (P < 0.05). In the upper segment samples, expression of sPLA2-IIA was significantly higher in the PTL group compared to the PTNL (P < 0.05). There was also a non-significant increase of sPLA2-IIA expression in TL compared to TNL samples (Figure 3b).

In both lower and upper segment myometrial samples, cPLA2-IV protein was most abundantly localized by immunohistochemical staining within the endothelial cells of blood vessels (Figure 4a). A faint and diffuse cytoplasmic distribution of cPLA2-IV expression was observed in lower segment myometrial smooth muscle cells.

Strong immunostaining for sPLA2-IIA was observed in both the endothelial and smooth muscle cells of the myometrial vessels (Figure 5a). In myometrial smooth muscle cells of lower segment samples, sPLA2-IIA tended to be localized on and around the nucleus, while in the upper segment myometrial smooth muscle, there appeared to be less nuclear but more cytoplasmic staining (Figure 5b LS and 5d US). Staining for sPLA2-IIA was also observed in lower segment stromal cells (Figure 5c) and in glandular epithelial cells of the upper segment (Figure 5e). In general the expression of sPLA2-IIA had a more distinct cellular localization compared to cPLA2-IV which was much more diffuse (except for that found around the periphery of the decidual cells). Exclusion of primary antibody, or pre-absorption with the antigen peptide, eliminated positive staining (Figures 4d–f and 5f–j).

In fetal membranes and placenta, expression of cPLA2-IV and sPLA2-IIA mRNA correlated with that of the protein, as determined by RT–PCR and western analysis respectively (n = 6 each). Expression of cPLA2-IV mRNA and protein was highest in the amnion and chorio-decidua compared to placenta (Figure 6a). By contrast, levels of sPLA2-IIA mRNA and protein were highest in chorio-decidua and placenta compared to the amnion (Figure 6b).

Discussion

We have demonstrated that expression of sPLA2-IIA is significantly up-regulated in pregnant compared to non-pregnant, and in labour, both at term and preterm, compared to non-labour myometrium. In contrast, cPLA2-IV expression was not significantly different in non-pregnant compared to pregnant or in labouring compared to non-labouring myometrium. We have also compared localization of cPLA2-IV and sPLA2-IIA between upper and lower segment myometrial samples.

Prostaglandins have long been implicated in the process of parturition. Inhibition of prostaglandin synthesis can lengthen gestation and delay labour in several species including humans (Lewis and Schulman, 1972). Previous studies have demonstrated that the COX-2 enzyme is up-regulated in fetal membranes and myometrium in late pregnancy and in association with labour (Hirst et al., 1995; Slater et al., 1995, 1999; Erkinheimo et al., 2000). However, prior to its utilization by COX, arachidonic acid must be released from membrane phospholipid stores by the action of phospholipases. Using RT–PCR to determine the relative abundance of cPLA2-IV protein was also found in decidual cells with a distinct pattern of staining observed around the periphery (Figure 4c).
and sPLA2-IIA, we have demonstrated increased expression of sPLA2-IIA, but not cPLA2-IV, in association with pregnancy and labour in human myometrium. This suggests that sPLA2-IIA, in addition to COX-2, may be one of the 'pro-labour' factors associated with late pregnancy, thus implying a role for sPLA2-IIA in the increased prostaglandin synthesis associated with the process of human parturition. By contrast, we were unable to detect any changes in the expression of cPLA2-IV mRNA either with increasing gestation or in association with labour. This is in concordance with data in the sheep whereby cPLA2-IV mRNA and protein were detected in myometrium but did not alter in association with labour (Zhang et al., 1996).

To verify whether the changes in mRNA are likely to predict changes in the corresponding protein, we examined the expression of both mRNA and protein by RT–PCR and western analysis respectively in fetal membranes and placenta. Levels of sPLA2-IIA mRNA were predictive of protein levels, with the highest expression in the placenta and chorio-decidua, compared to amnion. A positive

Figure 3. Type IIA secretory phospholipase A2 (sPLA2-IIA) mRNA expression in lower segment (left panel) and upper segment myometrium (right panel), during labour at term and preterm. Graph shows results expressed as mean and SE of individual ratio of densitometric units over glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Groups are preterm non-labour (PTNL) n = 7, preterm labour (PTL) n = 4, term non-labour (TNL) n = 14 and term labour (TL) n = 9. Significance was *P < 0.05 and **P < 0.005.

Figure 4. Representative immunolocalization of type-IV cytosolic phospholipase A2 (cPLA2-IV) protein in lower segment (a), (b), (d), and upper segment (c), myometrium at term. cPLA2-IV protein was localized to (a) endothelial cells of myometrial vessels, (b) myometrial smooth muscle cells and (c) decidual cells. No immunostaining was observed when myometrial tissue sections were incubated with pre-absorbed primary antibodies. Panels (d), (e) and (f) show control incubations of (a), (b) and (c) respectively.
correlation between mRNA and protein for cPLA2-IV was also shown, with expression highest in amnion and chorio-decidua and low in placenta. This positive correlation of mRNA and protein expression for both the sPLA2-IIA and the cPLA2-IV is important as it was not always possible to obtain enough myometrial tissue to analyse both mRNA and protein in the same samples. We are therefore confident that the increased expression of sPLA2-IIA mRNA observed during labour both at term and preterm in human myometrium is likely to result in increased expression of sPLA2-IIA protein.

No labour-associated changes of sPLA2-IIA expression have been shown in amnion, chorio-decidua or placenta (Munns et al., 1999); however, increased sPLA2-IIA levels may be associated with the spontaneous rupture of membranes (Lappas et al., 2001). Koyama et al. (2000) demonstrated increased levels of sPLA2-IIA in serum and amniotic fluid of women in preterm labour with and without chorio-amnionitis and suggested that measurement of sPLA2-IIA might be useful for preterm labour prediction. Indeed sPLA2 is considered a potent mediator of inflammation. It is up-regulated by cytokines, such as interleukin-1, which increases throughout gestation and in association with labour in fetal membranes (Keelan et al., 1999; Elliott et al., 2001) and amniotic fluid (Romero et al., 1992). Recent studies have demonstrated that inhibition of sPLA2-IIA mRNA expression, enzyme activity and prostaglandin production in placental explant cultures can be achieved using anti-sense oligonucleotides directed against the sPLA2 gene.

![Image of immunolocalization and blotting results](https://academic.oup.com/molehr/article-abstract/10/11/799/1113236/70x498 to 569x751)

Figure 5. Representative immunolocalization of type IIA secretory phospholipase A2 (sPLA2-IIA) protein in lower segment (a), (b), (c), and upper segment (d) and (e) myometrium at term. Panels (f), (g), (h), (i) and (j) show control incubations of (a), (b), (c), (d) and (e) respectively.

![Image of RT-PCR and western blotting results](https://academic.oup.com/molehr/article-abstract/10/11/799/1113236/143x104 to 496x283)

Figure 6. Representative data showing positive correlation of (a) type-IV cytosolic phospholipase A2 (cPLA2-IV) and (b) sPLA2-IIA mRNA with protein expression in fetal amnion (Am) and chorio-decidua (Ch) membranes and placenta (Pl) by RT–PCR and western blotting (c) shows glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT–PCR used to normalize mRNA levels.
(Lappas et al., 2001). Thus as myometrial sPLA2 mRNA levels are increased in association with preterm labour, the use of anti-sense technology for inhibition of this enzyme may provide novel therapeutic intervention.

Although we have identified that myometrial expression of sPLA2-IIA increases during labour, the question remains as to what is the precise function of the enzyme. Beside its role in the release of arachidonic acid for generation of prostaglandins and other second messengers, sPLA2-IIA has also been attributed to additional functions, including regulation of apoptosis, antiocoagulation, exocytosis and cell migration. It also has a number of antibacterial properties, apparently most effective against Gram-positive bacteria (review Kudo and Murakami, 2002; Lappas and Rice, 2004). It should be noted that whilst great care was taken to remove decidual and serosal components from myometrial biopsies using a dissection microscope, some of this tissue remained. Indeed, the immunohistochemistry results demonstrate that in addition to being localized in myometrial smooth muscle and endothelial cells of myometrial vessels, sPLA2-IIA was localized to glands and cPLA2-IV to decidual cells of the upper segment myometrium. Perhaps the answer, as to the role of sPLA2-IIA in human myometrial tissue, lies more with the precise cellular and intracellular localization of this enzyme. Production of sPLA2-IIA by endothelial cells of myometrial vessels may lead to increased prostacyclin production (Murakami et al., 1993), while the production of sPLA2-IIA by the glands in upper segment may have an exocrine role. The distinct localization of sPLA2-IIA to the nuclear and perinuclear region of myometrial smooth muscle cells has implications for downstream genomic effects. Further evidence in support of this is emerging, and suggests that a number of enzymes involved in prostaglandin synthesis, such as cPLA2, COX-1, COX-2, microsomal prostaglandin E2 synthase and possibly the prostaglandin receptors themselves, do localize to nuclear and perinuclear regions under various conditions and in distinct cell types. Taken together these data add to the complexity by which the various prostaglandins may elicit their effects (Bhattacharya et al., 1998; Freeman et al., 1998; Gobeil et al., 2003; Helliwell et al., 2004).

The precise function(s) of the different isoforms of sPLA2 within the uterus, is further complicated by the recent molecular cloning of a gene encoding a large sPLA2 receptor protein, which can exist as cPLA2, COX-1, COX-2, microsomal prostaglandin E2 synthase and possibly the prostaglandin receptors themselves, do localize to nuclear and perinuclear regions under various conditions and in distinct cell types. Taken together these data add to the complexity by which the various prostaglandins may elicit their effects (Bhattacharya et al., 1998; Freeman et al., 1998; Gobeil et al., 2003; Helliwell et al., 2004).

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