Lipoxygenase gene expression in baboon intrauterine tissues in late pregnancy and parturition

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Myometrium (upper and lower segment), cervix, chorion and decidua were obtained at the time of Caesarean hysterectomy in 15 baboons, 10 at varying gestational ages prior to the onset of labour in the last trimester of pregnancy, and five in spontaneous labour at term. Expression of currently recognized human lipoxygenase (LOX) genes was determined using Northern blot analysis relative to each of three housekeeping genes. Signals of similar size to human 5-LOX and human platelet 12-LOX genes were detected in all tissues. Expression of 5-LOX mRNA in the cervix decreased with advancing gestational age. In decidua, expression of 5-LOX mRNA was higher in tissues from animals in labour (compared to those not in labour), whereas in chorion, its expression was lower in tissues from animals in labour. Expression of the platelet 12-LOX gene decreased in chorion with advancing gestational age, and in cervix was lower in tissues from animals in labour. We postulate that the variation in expression of LOX genes may play a role in the onset or promotion of parturition in the baboon.

Key words: baboon (Papio) / gene expression / lipoxygenase / parturition / pregnancy

Introduction

Several studies have underlined the key role of prostaglandins (PG) in the control of parturition in a number of species (Challis et al., 1997). PG are formed from arachidonic acid by the two isoforms of the enzyme cyclo-oxygenase (COX). Expression of the inducible form of this enzyme (COX-2) has been shown to increase both with advancing gestational age and labour (Zhang et al., 1996; Slater et al., 1999a,b; Wu et al., 1999; Erkinheimo et al., 2000) and these studies have led directly to clinical studies of selective COX-2 inhibitors in the clinical management of preterm labour (Sawdy et al., 1997). However, it has long been recognized that there are at least two other major enzymatic pathways of arachidonic acid metabolism which result in biologically active products, namely, the epoxide pathway (cytochrome P450), and the lipoxygenase (LOX) pathway (Campbell and Halushka, 1996). The latter generates hydroperoxyeicosatetraenoic acid (HETE) compounds which are metabolized to a range of eicosanoids, including the leukotrienes (LT) and some of these have been shown to stimulate myometrial contractility (Bennett et al., 1987). A wide range of drugs have been developed to either inhibit LOX enzymes or block the receptors through which LOX products act (Coleman et al., 1995; Drazen et al., 1999). If LOX products have a role in parturition, there is the potential for these drugs to be employed in the clinical management of premature labour.

The three main LOX genes identified in the human are those encoding 5-LOX, platelet 12-LOX and 15-LOX (Funk, 1996). In the mouse there is another isoform, namely, leukocyte 12-LOX. However, this enzyme also has 15-LOX activity and shares 65% amino acid homology with human 15-LOX (Funk, 1996) and it is thought that leukocyte 12-LOX is the murine homologue of human 15-LOX. Previous studies have shown that labour is associated with an increased expression of the 5-LOX gene in human choriondecidua (Brown et al., 1999) and that amniotic fluid levels of 5-LOX products are increased in labour (Romero et al., 1988). Some lines of evidence suggest a causal role for increased LOX expression in the initiation of parturition, specifically, amniotic fluid levels of 5-LOX products increase prior to the onset of labour in rhesus macaques (Haluska et al., 1990) and failed induction of labour is associated with decreased 5-LOX expression (Faber et al., 1996). However, other findings suggest that increased expression of LOX genes and release of LOX products may simply be a response to parturition since LOX products are released from critical intrauterine tissues in response to oxytocin (Pasetto et al., 1992), activation of protein kinase C (Edwin and Mitchell, 1994) and calcium influx (Edwin et al., 1995).

We sought to determine the relationship between LOX gene expression and both gestational age and labour. Since critical human tissues such as cervix and fundal myometrium are
difficult to obtain from pregnant women, we performed Caesarean section hysterectomy on 15 pregnant baboons, 10 at various stages in the last trimester of pregnancy but not in labour, and five animals in spontaneous labour at term. We quantified expression of the three currently recognized human LOX genes in myometrium (both fundus and lower segment), cervix, decidua and chorion.

Materials and methods

Animal methods

Animal methods and surgical procedures have been described 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. The uterus and its contents were removed at total Caesarean hysterectomy under general anesthesia (ketamine induction, halothane maintenance).

At the time of surgery, 10 animals were not in labour and in the last trimester of pregnancy at the following days gestational age (days gestational age): 121, 128, 141, 153, 159, 162, 162, 177, 177, 180. (term is ~185 days). The cervix was uneffaced 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 days gestational age). Analysis of electromyogram (EMG) traces of the 48 h preceding delivery revealed that myometrial activity was solely of the contractures type with no contraction activity. No drugs of any form had been administered to any of the animals in the 2 weeks preceding surgery. Caesarean hysterectomy was also performed on five animals in spontaneous labour. Of these, four had EMG electrodes sited. These animals had a baseline cervical examination at the time of their first EMG contraction activity and were re-examined when they had a sustained switch from contractures to contractions (>30 min). The gestational ages (in days) at hysterectomy were 164, 184, 191 and 193. The cervical dilations for these animals were 6, 3, 3 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 days gestational age and, at the time of the procedure, it was found that the cervix was 4 cm dilated and fully effaced and a hysterectomy was performed.

The hysterectomies from the pool of animals not in labour were performed over a 2 year period. Some of the hysterectomies were performed after some of the initial blots were obtained, but samples were run for Northern analysis from at least seven of the animals not in labour. All analyses include samples from all five animals in labour at the time of hysterectomy.

All tissues were flash-frozen in liquid nitrogen and stored at ~80°C until use. The chorion and amnion were separated. Decidua was dissected from the uterine cavity following removal of the fetal membranes. The cervix was dissected immediately following removal of the uterus and myometrial specimens were obtained following complete removal of decidua. The lower segment was defined as the portion of the uterus 1 cm immediately superior to the internal os of the cervix. The fundus was defined as the portion of the uterus superior to the upper limit of the uterine cavity. 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 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, 4 or 5 µg) were denatured in 17.4% (vol/vol) formaldehyde, 50% (vol/vol) freshly deionized formamide, 20 mmol/l MOPS [3-(N-morpholino) propanesulphonic acid], 5 mmol/l sodium acetate and 1 mmol/l EDTA, pH 7.0, for 5 min at 65°C and separated by electrophoresis on a 1.4% (wt/vol) agarose-0.66 mol/l formaldehyde gel (containing 0.28 µg/ml ethidium bromide). The gel was visualized under UV transillumination and photographed, both to confirm the integrity of RNA and to determine the distance of migration of a series of standard RNA markers (Gibco, Rockville, MD, USA). The RNA was transferred onto a nylon membrane (Gene Screen plus; NEN; DuPont, Wilmington, DE, USA) by capillary blotting for 24 h in 10×SSC (1×SSC is 0.15 mol/l NaCl and 0.015 mol/l sodium citrate, pH 7.0). Prehybridization (>1 h) and hybridization (>18 h) were carried out in hybridization bottles in an oven at 65°C when using riboprobes and 45°C in sealed bags when a cDNA probe was used. A commercial 50% formamide-based hybridization buffer was employed (Northern Max or UltraHyb; Ambion, Austin, TX, USA). The probe concentration was ~1×10⁶ cpm/ml of hybridization buffer. Membranes were washed twice for 5 min in 2×SSC and 0.1% at 65°C and twice for 1 h 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

Antisense riboprobes were synthesized using a commercial kit (StripEZ RNA; Ambion) and labelled with [α-32P]UTP (800 Ci/mmol) (NEN Life Science). Template DNA was removed by addition of 2 U of RNase-free DNase and incubation for 15 min at 37°C. cDNA probes were labelled with [α-32P]ddeoxy-CTP (3000 Ci/mmol) using the random priming method (StripEZ DNA; Ambion) to specific activities of ~1×10⁹ c.p.m./µg. Both DNA and RNA probes were separated from unincorporated nucleotide using a spin column (Probe Quant G-50; Pharmacia Amersham, Piscataway, 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 for each of three housekeeping genes.

The cDNA for human 5-LOX, platelet 12-LOX, and 15-LOX were all purchased from Oxford Biomedical (Oxford, MI, USA). The plasmids (all TRIscript; Ambion) containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and cyclophilin cDNA with RNA polymerase promoters were purchased from Ambion.

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 a plot of the distance of migration plotted versus molecular weight of the markers. The intensity of autoradiographic signals was quantified using densitometry. The expression of a given LOX gene was expressed as a ratio to each of three housekeeping genes: GAPDH, β-actin and cyclophilin. Correlation was determined using simple linear regression. Comparison of two means was made by Student’s t-test. Comparison of three or more means was performed using analysis of variance and a repeated measures model was employed where more than one value was obtained from each animal. Comparison of means adjusted for gestational age was performed using analysis of covariance. Statistical analysis was performed using
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Stata, version 6.0 for Windows. Statistical significance was assumed at the 5% level.

Results

Clear signals of similar size to previous reports of Northern analysis of human RNA were detected using the 5-LOX and platelet 12-LOX probes in myometrium (Figure 1), cervix (Figure 2), decidua (Figure 3) and chorion (Figure 4). In the case of 5-LOX there was a single band of ~2.4 kb. In the case of platelet-12-LOX there was a single band of 2.5 kb in decidua, but in myometrium, cervix and chorion, there was an additional band at 2.2 kb. A clear 6.3 kb band was observed using the human 15-LOX cDNA probe in myometrium and cervix (Figure 5). However, this differs markedly in size from previous reports of the 15-LOX gene in Northern analysis of human tissues (Conrad et al., 1992) and it remains to be determined whether this signal truly represents the baboon 15-LOX gene. This signal was not observed in either decidua or chorion. The yield of mRNA from amnion was too small to permit Northern analysis of expression of any of the LOX genes.

Densitometric analysis (relative to β-actin) showed that with advancing gestational age, there was decreased expression in the cervix of the 5-LOX gene ($r^2 = 0.7$, $P = 0.02$) and decreased expression of the 2.5 kb transcript of the platelet 12-LOX gene in chorion ($r^2 = 0.7$, $P = 0.02$) (Figure 6). There was no other significant correlation with gestational age among any of the transcripts in any of the other tissues (all $P > 0.05$).

Densitometric analysis (relative to β-actin) showed that myometrial expression was not significantly different when comparing samples obtained from the fundus and lower segment either in labour or not in labour for either 5-LOX or platelet 12-LOX (all $P > 0.05$). The expression of 5-LOX and the 2.2 kb platelet 12-LOX transcript did not vary significantly with labour in the cervix ($P > 0.05$). However, expression of

Figure 1. Northern blot of LOX expression in myometrium from lower uterine segment (LUS) and fundus (FUN), from animals not in labour (NIL) and in labour (LAB). (A) 5-LOX, (B) platelet 12-LOX. The estimated size of transcripts is given in kilobases (kb). 2 µg of RNA was loaded in each lane. Lanes: (A) 1–10 LUS, NIL (in order of gestational age, i.e. earliest gestation: lane 1); 11–15 LUS, LAB; 16–25 FUN, NIL (in order of gestational age); and 26–30 FUN, LAB. (B) 1–9 LUS, NIL (in order of gestational age); 10–14 LUS, LAB; 15–23 FUN, NIL (in order of gestational age); 24–28 FUN, LAB. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
the 2.5 kb platelet 12-LOX transcript was lower among animals in labour in the cervix (Figure 7A). In decidua, labour was associated with a higher level of expression of the 5-LOX gene (Figure 7B), but there was no significant variation in platelet 12-LOX expression ($P > 0.05$). In chorion, labour was associated with a lower level of expression of the 5-LOX gene (Figure 7C), but there was no significant variation in platelet 12-LOX expression ($P > 0.05$).

There was no significant difference in the expression of the 6.3 kb transcript detected using the 15-LOX probe (relative to β-actin) when comparing fundus and lower segment ($P > 0.05$) and when comparing tissues from animals in labour and not in labour in fundus, lower segment and cervix (all $P > 0.05$).

All cases where significant variation was observed when signals were related to β-actin were also significant when related to at least one of the other two housekeeping genes (data not shown). Where signals were observed to change with gestational age prior to labour (namely, 5-LOX in the cervix and platelet 12-LOX in the chorion) adjusting the comparison between animals in labour and not in labour for the effect of gestational age (using analysis of covariance) did not alter the statistical significance.

**Discussion**

The estimated molecular weight of transcripts observed in the present study using the human 5-LOX and platelet 12-LOX probes were within 0.1 kb of previous reports of Northern analysis in human tissues (Matsumoto *et al.*, 1988; Funk *et al.*, 1992). We are confident, therefore, that these signals are likely to represent the baboon transcripts of these genes. However, the transcript observed using the 15-LOX probe was approximately twice as large as previous descriptions of Northern blot of human tissue (Conrad *et al.*, 1992). We have reported our findings with this probe for completeness (Figure 5), but suggest that the results be interpreted with some caution until more is known about the baboon 15-LOX gene.

**Figure 2.** Northern blot of LOX expression in cervix from animals not in labour (NIL) and in labour (LAB). (A) 5-LOX, (B) platelet 12-LOX. The estimated size of transcripts is given in kilobases (kb). 4 µg of RNA loaded in each lane in (A) and 2 µg loaded in each lane in (B). Both blots, lanes 1–7 NIL (in order of gestational age) and lanes 9–13 LAB. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

**Figure 3.** Northern blot of LOX expression in decidua from animals not in labour (NIL) and in labour (LAB). (A) 5-LOX, (B) platelet 12-LOX. The estimated size of transcripts is given in kilobases (kb). 5 µg of RNA loaded in each lane. Lanes: (A) 1–8 NIL (in order of gestational age), 9–13 LAB; (B) 1–9 NIL (in order of gestational age), 10–14 LAB. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
Changes were observed in the expression of LOX genes which both preceded labour (i.e., variation in expression in the last trimester of pregnancy) and occurred in association with labour. Decreased expression of LOX genes in the last trimester of pregnancy may have a role in the initiation of parturition and these findings are consistent with studies in the sheep which have demonstrated a decreased ratio of LOX:COX products with advancing gestational age (Langlois et al., 1993). Changes in LOX gene expression were also observed in association with labour, but it remains to be determined whether these changes have a causal role in the initiation of parturition or whether they are a consequence of other factors which are initiating and promoting parturition.

The changes observed in expression of 5-LOX were tissue specific. Expression of the gene decreased with advancing gestational age in cervix and was lower in chorion obtained from animals in labour. However, 5-LOX expression was greater in deciduala obtained from animals in labour. Increased decidual 5-LOX expression suggests that 5-HPETE or its metabolites might have a role in promoting parturition. This could either be due to autocrine effects on the decidua or paracrine effects on myometrium. Consistent with this, 5-HETE has been shown to stimulate contractility in myometrial strips obtained from the lower uterine segment of pregnant women (Bennett et al., 1987). The decrease in 5-LOX expression in cervix and chorion may indicate that in these tissues, 5-HPETE or its metabolites may act to inhibit the process of parturition. Consistent with this hypothesis, leukotriene B4 (LTB4) has been shown to exert inhibitory effects on cervical tissue obtained from pregnant women at term (Bryman et al., 1985). An alternative possibility is that 5-HPETE and its metabolites are without significant effects in these tissues and that the main biological consequence of lower levels of 5-LOX expression may be to divert free arachidonic acid to the PG pathway. These mechanisms may also act additively, since exogenous LTB4 has been shown to inhibit PGE2 release in human fetal membranes (Ticconi et al., 1995). Therefore, decreased 5-LOX may stimulate PG release due to both an increase in the free arachidonic acid pool and direct effects of LT on PG biosynthesis.

The size of the platelet 12-LOX transcript observed in the baboon (2.5 kb) was very similar in size to the human transcript (2.4 kb) which supports the interpretation that this signal is likely to represent the platelet 12-LOX gene in the baboon (Funk et al., 1992). However, in myometrium, cervix and chorion a second transcript of 2.2 kb was also observed. The level of expression of the smaller transcript was lower than the 2.5 kb transcript and quantitative analysis of the level of expression was only possible in cervix. Labour appeared to differentially affect expression of the two transcripts in the cervix.

We postulate that the multiple transcripts observed represent alternative mRNA splicing of the baboon platelet 12-LOX gene. Previous studies of LOX genes have demonstrated alternatively spliced isoforms with tissue specific variation in expression (Thiele et al., 1999). Furthermore, these experiments have suggested that variation in the 3' untranslated regions of mRNA transcripts might regulate LOX gene translation. The observed variation in the relative expression of the 2.5 kb and 2.2 kb platelet 12-LOX transcripts are, therefore, consistent with a role for platelet 12-LOX in the control of parturition in these tissues. Clearly, however, further studies of the baboon platelet 12-LOX gene will be required to clarify the significance of these observations.

If variation in translation of functional platelet 12-LOX is confirmed, it may reflect effects of 12-HPETE (or its metabolites) on key intrauterine tissues. However, it has been shown that 12-HETE has no effect on the contractility of isolated strips of human myometrium (Bennett et al., 1987). As discussed above in relation to 5-LOX, it may be that reduced platelet 12-LOX activity may divert free arachidonic acid to COX and might be a factor in determining increased PG levels in labour, amplifying the effect of other known factors which increase PG synthesis in labour, such as increased expression of phospholipase A2 and COX-2 (Zhang et al., 1996).

Comparing the findings of this study with other studies, it has also been shown in the human that mRNA encoding the 5-LOX gene increases in the choriodecidua in association with

Figure 4. Northern blot of LOX expression in chorion from animals not in labour (NIL) and in labour (LAB). (A) 5-LOX, (B) platelet 12-LOX. The estimated size of transcripts is given in kilobases (kb). 5 μg of RNA loaded in each lane. Both blots, lanes 1–7 NIL (in order of gestational age) and lanes 9–13 LAB. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
labour and this was paralleled by increased 5-LOX protein (Brown et al., 1999). This is consistent with the observation that amniotic fluid concentrations of both 5-HETE and LTC4 are higher in samples obtained from women in labour compared with women not in labour (Romero et al., 1988, 1989). The finding that 5-LOX and platelet 12-LOX are the main LOX genes expressed in intrauterine tissues is consistent with biochemical studies which have demonstrated that 5-HETE and 12-HETE are the main LOX products of arachidonic acid in human intrauterine tissues (Saeed and Mitchell, 1983; Schafer et al., 1996). Our hypothesis that decreased 12-HETE formation in labour may play a role in increasing free arachidonic acid and thus promoting formation of PG is supported by the observation that 12-HETE is quantitatively the major arachidonic acid metabolite formed in key intrauterine tissues (Schafer et al., 1996) and that human labour is associated with an increased ratio of COX:LOX metabolites of arachidonic acid (Bennett et al., 1993).

There were no apparent differences in myometrial expression of LOX genes comparing the upper and lower segment of the uterus. This is in contrast to COX genes which have been shown to be differentially expressed according to the uterine site in the baboon (Wu et al., 2000). These observations also tend to support an interpretation of different physiological roles for COX and LOX products in the control of parturition. However, we cannot exclude significant variation in LOX genes in amnion in association with labour. The yield of RNA from amnion was too small to study LOX expression using the methods employed in this study. Previous studies in the human have confirmed 5-LOX expression in the amnion but have not demonstrated any significant increase with advancing gestational age or labour (Brown et al., 1999).

These data do not strongly suggest, at present, a potential role for LOX inhibitors in the treatment of preterm labour. Indeed, the observations that both advancing gestational age and labour are associated with decreases in the expression of

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**Figure 5.** Northern blot of 15-LOX expression in (A) myometrium, (B) cervix. 2 µg of RNA loaded in each lane. Samples of myometrium were from both lower uterine segment (LUS) and fundus (FUN), and samples of both tissues were obtained from animals not in labour (NIL) and in labour (LAB). The estimated size of transcripts is given in kilobases (kb). Lanes: (A) 1–9 LUS, NIL (in order of gestational age); 10–14 LUS, LAB; 15–24 FUN, NIL (in order of gestational age); and 25–29 FUN, LAB. (B) 1–7 NIL (in order of gestational age) and 9–13 LAB. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
some LOX genes in key tissues suggests that pharmacological inhibition of LOX genes may actually promote parturition, possibly by diverting free arachidonic acid to the PG pathway. However, the increased expression of 5-LOX in decidua is consistent with a possible role for 5-LOX products (such as LT) in parturition. Multiple receptors exist for these compounds and drugs with selective antagonist activity have been described (Coleman et al., 1995). Unlike enzyme inhibitors, receptor antagonists would not be expected to increase the pool of free arachidonic acid and the effect of the available drugs on parturition in animals warrants further study.

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References


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