Characterization of topology-, gestation- and labor-related changes of a cassette of myometrial contraction-associated protein mRNA in the pregnant baboon myometrium

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Abstract

Our objective was to examine the topology-, gestation- and labor-related changes of estrogen receptor (ER) α , progesterone receptor (PR), oxytocin receptor (OTR) and thrombospondin-1 (TSP1) mRNA in pregnant baboon myometrium.

ER α , PR, OTR and TSP1 mRNAs extracted from the lower uterine segment and fundal myometrium of pregnant baboons not in labor between 121 and 180 days of gestational age ($n=9$) and in established spontaneous labor between 164 and 193 days of gestational age ($n=5$) were analyzed by Northern blot.

There were no topology-, gestation- or labor-related changes of ER α and PR mRNA in or between the lower uterine segment or/and the fundus. OTR mRNA was the same in the lower uterine segment and the fundus from baboons not in labor and non-labor fundal, but not lower uterine segment, myometrial OTR mRNA increased with gestation ($R^2=0.81$, $P<0.05$). Fundal OTR mRNA

rose significantly compared with the lower uterine segment during spontaneous labor. TSP1 mRNA increased significantly in both the fundus and lower uterine segment during labor. TSP1 mRNA in the lower uterine segment during spontaneous labor was higher than in the fundus during spontaneous labor.

In conclusion, fundal and lower uterine segment ER α and PR mRNA remained unchanged in late gestation and spontaneous labor. The increased OTR mRNA may serve as a mechanism to increase uterine sensitivity to OT during late gestation. The higher fundal OTR mRNA compared with the lower uterine segment provides polarity which assists fetal expulsion by uterine contractions during labor. The significance of increased TSP1 mRNA during labor may relate to homeostasis and merits further study.

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Introduction

Preparation of the myometrium associated with labor involves the appearance of a cassette of myometrial contraction-associated proteins (mCAPs). The genes for these proteins are up-regulated or down-regulated at labor (Lye 1994, Zingg *et al.* 1995, Wu *et al.* 1998). Each member of the cassette of genes contributes to some extent to the switch in myometrial contractility patterns from contractures to contractions as well as the progression of established labor. The nature of the change in myometrial contractile patterns differs from species to species. In sheep, one of the most extensively studied experimental models, myometrial activity switches from contractures to contractions only once, 12–24 h before delivery. In contrast, in non-human primates and pregnant women, the switch from contractures to contractions occurs at night and generally recurs for several nights before delivery (Taylor *et al.* 1983). Altered abundance of members of this

cassette of genes in the myometrium is a prerequisite for normal labor and delivery. Myometrial activation before and during labor appears to play a critical role in connecting fetal signals that indicate fetal readiness for birth to maternal factors that carry labor forward to produce safe delivery of the fetus. It is important to evaluate these events in non-human primates as well as sheep.

Healthy uterine tissues obtained at precise times in relation to the onset of labor and suitably processed for study are required to permit evaluation of molecular changes associated with labor. It is difficult to obtain the necessary range of hysterectomy specimens from healthy pregnant women. To obtain information regarding the topology of the critical mCAPs it is necessary to obtain tissues from different regions of the uterus. There are now increasing data from both human and animal studies to suggest that the expression of a range of mCAPs is spatially regulated within the uterus (Fuchs *et al.* 1984, Smith *et al.* 1998, Sparey *et al.* 1999). For all of these reasons, normal

non-human primate tissues are indispensable to a better understanding of human parturition. The baboon and other non-human primates share similarities with pregnant women in their reproductive anatomy, physiology and endocrinology throughout pregnancy and during labor.

Both estrogen and progesterone play important roles in preparing the myometrium for labor (Challis & Lye 1994, Nathanielsz *et al.* 1994, 1998). The balance between estrogen and progesterone is a key factor that maintains uterine quiescence (Csapo 1975) throughout pregnancy. In a large number of different species, it has been clearly shown that increased estrogen production is associated with delivery (Nathanielsz *et al.* 1994, 1998). Infusion of androgen to pregnant rhesus monkeys in late gestation to provide a precursor for placental estrogen synthesis will initiate premature delivery (Mecenas *et al.* 1996). Inhibition of androgen conversion to estrogen will inhibit premature delivery (Nathanielsz *et al.* 1998). The effects of estrogen and progesterone are mediated through estrogen and progesterone receptors (Carson-Jurica *et al.* 1990). Alterations in estrogen receptor (ER) and progesterone receptor (PR) concentrations may also play an important role in the changing responses of the myometrium to estrogen and progesterone.

The oxytocin (OT) receptor (OTR) is another important mCAP which has been studied extensively in association with labor in the rat (Zingg *et al.* 1995, Ou *et al.* 1998), sheep (Wu *et al.* 1996c), and pregnant women (Fuchs *et al.* 1984). No information is available on the important issue of precise topological distribution of OTR in the non-human primate uterus. Such information is of considerable value for understanding OT physiology in human parturition. Our own studies in the rhesus monkey (Giussani *et al.* 1996) and baboon (Nathanielsz *et al.* 1997), and those of others in pregnant women (Goodwin *et al.* 1996) clearly demonstrate a role for OT during labor. In pregnant women, increased sensitivity of the pregnant uterus to OT parallels an increased concentration of myometrial OTR (Zeeman *et al.* 1997).

We have previously used suppression subtractive hybridization to identify a parturition-related increase in mRNA and protein in one extracellular matrix protein, thrombospondin-1 (TSP1), in the pregnant ovine myometrium (Wu *et al.* 1999a). Our data suggest that changes in uterine extracellular matrix proteins might also play an important role associated with myometrial activation during labor. We therefore determined whether similar changes in TSP1 mRNA occurred in the pregnant baboon myometrium associated with the onset of labor.

Materials and Methods

Animals

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 comparing ultrasonography measurements with records of sex skin swelling. Cesarean section hysterectomy was performed with the animals under halothane general anesthesia in nine baboons not in labor at the following gestational ages: 121, 146, 153, 158, 162, 162, 177, 177, and 180 days (term 175–178 days) and eight baboons in spontaneous labor at the following gestational ages: 164, 172, 184, 191, and 193 days. Samples of myometrium were obtained from different regions of the uterus. Uterine electromyographic (EMG) leads had been previously sited in three of eight animals not in labor and the five baboons in spontaneous labor. Animal care and EMG recording and analysis have been described elsewhere (Morgan *et al.* 1992). Myometrial activity was monitored continuously and evaluated as being either in the low amplitude, infrequent contracture mode (not in labor group), or in the frequent short duration labor type contraction mode (spontaneous labor group) with cervical effacement and dilation. No drugs in any form had been administered to any of the animals in the 2 weeks before surgery. 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.

Samples were dissected immediately after removal of the uterus and flash frozen for later extraction of RNA. The fundus was defined as the portion of the uterus immediately superior to the upper limit of the uterine cavity. The lower uterine segment was defined as the portion of the uterus superior to the internal os of the cervix. The upper limit of the lower uterine segment was a line 1 cm superior to the internal os of the cervix and the lower limit was the internal os itself.

Northern blot analysis

Poly-A RNA was extracted from flash-frozen myometrial samples stored at -80°C by oligo dT cellulose affinity chromatography using a commercial kit (Fast Track 2.0; Invitrogen, San Diego, CA, USA). The poly-A RNA purity and recovery of each tissue was determined by UV spectrophotometer (260 and 280 nm). There were no differences in the yield of RNA per mg tissue between baboons in labor and baboons not in labor.

Samples of poly-A RNA (2 $\mu\text{g}/\text{lane}$) from each tissue were denatured in 17.4% (v/v) formaldehyde, 50% (v/v) formamide, 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA, pH 7.0 for 5 min at 65°C and separated on a 1% (w/v) agarose/0.66 M formaldehyde gel. After electrophoresis, RNA was transferred to a nylon membrane (Gene Screen Plus; New England Nuclear, Dupont, Wilmington, DE, USA) by capillary blotting for 24 h in $10 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl and 0.015 M Na citrate, pH 7.0).

Figure 1 (A) Northern analysis of ERα mRNA in non-labor (NL) lower uterine segment (LUS; lanes 1–9) and NL fundus (FUN; lanes 15–23) compared with spontaneous labor (SL) LUS (lanes 10–14) and SL FUN (lanes 24–28). (B) The same blot was probed for β-actin. (C) Densitometric analysis of the ratio of ERα and β-actin mRNA in NL LUS and NL FUN (means ± S.E.M.; $n=9$ for both groups) compared with SL LUS and SL FUN (means ± S.E.M.; $n=5$ for both groups). There were no changes of ERα between animals in NL and SL and there were no changes of ERα distribution between LUS and FUN.

Membranes were prehybridized at 42 °C for 5 h in hybridization solution (50% (v/v) deionized formamide, 50 mM sodium phosphate, 0.8 M NaCl, 2% (w/v) sodium dodecyl sulphate (SDS), 100 µg salmon sperm DNA/ml, 20 µg tRNA/ml and 1 × Denhardt's (50 × = 1% solution of bovine serum albumin, Ficoll, and polyvinylpyrrolidone).

A 131 bp cDNA probe encoding part of the sheep endometrial OTR, which was generated by PCR, was kindly made available to us by Dr A P F Flint (University of Nottingham, Nottingham, Notts, UK). Recombinant human ERα cDNA containing the entire coding region was kindly made available by Dr P Chambon (University of Strasbourg, Strasbourg, France). Human PR cDNA probe was made available to us by Dr B W O'Malley (Baylor College of Medicine, Houston, TX, USA). Ovine

TSP1 cDNA probe was cloned in our laboratory (Wu *et al.* 1999a). The cDNA probes were labeled with α³²P dCTP using the random priming method (NEN-Dupont) to specific activities of approximately 1 × 10⁹ c.p.m./µg. Labeled cDNA was utilized at a final concentration of 1 × 10⁶ c.p.m. specific probe/ml hybridization solution.

Hybridization was carried out at 42 °C for 20 h. Membranes were washed sequentially in 2 × SSC at room temperature for 10 min, and 0.2 × SSC with 0.1% SDS at 65 °C for 30 min. Kodak X-OMAT AR film was exposed to the membrane with intensifying screens at –80 °C. Exposure durations were varied to achieve hybridization signals within the limited linear range for densitometry. After probing for the receptor gene of interest, membranes were rehybridized with a α³²P-labeled cDNA probe encoding β-actin to normalize each of ERα, PR,

Figure 2 (A) Northern analysis of PR mRNA in non-labor (NL) lower uterine segment (LUS; lanes 1–9) and NL fundus (FUN; lanes 15–23) compared with spontaneous labor (SL) LUS (lanes 10–14) and SL FUN (lanes 24–28). (B) The same blot was probed for β-actin. (C) Densitometric analysis of the ratio of PR and β-actin mRNA in NL LUS and NL FUN (means $\pm$ S.E.M.; $n=9$ for both groups) compared with SL LUS and SL FUN (means $\pm$ S.E.M.; $n=5$ for both groups). There were no changes of PR during SL and there were no changes of PR distribution between LUS and FUN.

OTR and TSP1 mRNA levels. Poly-A gel stained with ethidium bromide was also used to monitor the poly-A RNA loading. Autoradiograph signals were analyzed and quantified with a scanner (ScanMaker II; Microtek, Pt Washington, NY, USA) and data were analyzed in a power Macintosh computer (8100/80) with a densitometry program (Scan Analysis; Biosoft, Cambridge, Cambs, UK).

Statistical analysis

Comparison of two means was made with the Student's *t*-test. Comparison of three or more means was made by analysis of variance, and multiple post hoc comparisons were made with Tukey's method for estimating the 95%

confidence interval of pairwise differences. Statistical significance was assumed at the 5% level. The changes associated with gestation were analyzed by regression analysis. Data are presented throughout as means $\pm$ S.E.M.

Results

The expression of ERα and PR in the myometrium

Northern blot analysis of poly-A RNA from the pregnant baboon myometrium revealed a single transcript for ERα and PR with approximate molecular weights at 6.2 kb (Fig. 1) and 11.0 kb (Fig. 2) respectively. There were no gestational- or labor-related changes for ERα and PR in either the lower uterine segment or the fundus (Figs 1 and

Figure 3 (A) Gestational-associated changes in the abundance of OTR mRNA in non-labor fundus (lanes 1–8) obtained from baboons at the following gestational age in days (dGA): 121 (lane 1), 153 (lane 2), 158 (lane 3), 162 (lane 4), 162 (lane 5), 177 (lane 6), 177 (lane 7), and 180 (lane 8). (B) The same blot was rehybridized with a β -actin probe to demonstrate relative amounts of RNA in each lane. (C) Densitometric analysis of the ratio of OTR and β -actin mRNA as a function of the gestational age. There was a significant increase of OTR mRNA in non-labor fundus associated with increased gestational age ($P < 0.01$).

2). In addition, there was no differential regional distribution of ER α and PR in the lower uterine segment compared with the fundus (Figs 1 and 2).

The expression of OTR in the myometrium

There was a major RNA transcript at 6.6 kb similar to the size of the ovine OTR transcript (Wu *et al.* 1996c), which hybridized with the OTR cDNA probe (Figs 3 and 4). There was a significant increase of non-labor fundal, but not lower uterine segment, myometrial OTR mRNA associated with increased gestational age ($R^2 = 0.81$, $P < 0.01$, Fig. 3). OTR mRNA in myometrium was significantly higher in the fundus when compared with the lower uterine segment during spontaneous labor but the difference was not significant prior to labor. Since there was no increase in the fundus as a result of labor the difference between the lower uterine segment and the fundus at the time of labor likely reflects, at least in part, a fall in OTR mRNA in the lower uterine segment (Fig. 4).

The expression of TSP1 in the myometrium

TSP1 mRNA was well expressed in the pregnant baboon myometrium as an approximate 6.5 kb TSP1 mRNA transcript in the myometrium (Fig. 5). There were no gestational-related increases for TSP1 mRNA in either lower uterine segment or fundus (Fig. 5). TSP1 mRNA increased significantly in both fundus and lower uterine segment myometrium during labor and fundal TSP1 mRNA was significantly lower than the lower uterine segment during labor (Fig. 5).

Discussion

The balance of the action of estrogen and progesterone is important in regulating the extensive morphological, biochemical and physiological changes in the pregnant uterus. These steroids are also essential to suppress myometrial activity, control uterine blood flow and maintain the pregnancy as well as to switch the myometrial contraction patterns that occur at the end of pregnancy (Nathanielsz 1998). Effects of estrogen and progesterone are dependent upon the expression of ER and PR. There are two subtypes of ER, ER α and ER β (Kuiper *et al.* 1996). The different receptor subtypes may have tissue-specific and physiological-state specific actions. The present study focused on ER α , the 'classical' form of ER (Green *et al.* 1986).

The current study is the first to examine topological-, gestational- and labor-associated changes of the ER α and PR mRNA in the pregnant baboon myometrium. There were no changes of ER α in the pregnant baboon myometrium associated with gestation and labor. This observation is consistent with previous reports in rhesus monkey myometrium analyzed by binding assay (Novy & Haluska 1994) and immunohistochemistry (Haluska *et al.* 1990) and our own data obtained by RT-PCR (Wu *et al.* 2000).

In contrast, a dramatic increase of ER α mRNA occurs in sheep associated with both spontaneous term labor (Wu *et al.* 1995) and glucocorticoid-induced premature labor (Wu *et al.* 1996a). We and others have clearly demonstrated that estradiol up-regulates ER α expression and progesterone down-regulates ER α expression in non-pregnant uterine tissues in sheep (Wu *et al.* 1996b), mouse (Bergman *et al.* 1992), rhesus monkey (Okulicz *et al.* 1993) and a human breast cancer cell line (Cho *et al.* 1991). In addition, the observation that uterine ERs are increased by the PR antagonist RU486 in late gestation in the pregnant rhesus monkey is another example of progesterone's suppressive effect on uterine ER α (Haluska *et al.* 1990). These results suggest that the control of uterine ER α is dependent on the complex interplay of estrogen and progesterone. In sheep, the sharply increased maternal plasma estradiol concentration (Challis 1971) and decreased maternal plasma progesterone (Thorburn *et al.* 1988) that occur immediately before parturition may together be

Figure 4 (A) Northern analysis of OTR mRNA in non-labor (NL) lower uterine segment (LUS; lanes 1–9) and NL fundus (FUN; lanes 15–23) compared with spontaneous labor (SL) LUS (lanes 10–14) and SL FUN (lanes 24–28). (B) The same blot was probed for β -actin. (C) Densitometric analysis of the ratio of OTR and β -actin mRNA in NL LUS and NL FUN (means $\pm$ S.E.M.; $n=9$ for both groups) compared with SL LUS and SL FUN (means $\pm$ S.E.M.; $n=5$ for both groups). OTR mRNA was the same in NL LUS and NL FUN. However, there was a significant increase in OTR mRNA in FUN compared with LUS during SL (* $P<0.05$).

responsible for increased expression of uterine ER α during labor. In contrast, in non-human primates and pregnant women, the increase of estrogen throughout late gestation is gradual and progesterone concentration in maternal plasma is maintained during labor (Simpson & MacDonald 1981). These differences in maternal hormone environment may explain the interesting species differences we describe here in ER α mRNA during pregnancy and labor in the baboon.

Much less is known about physiological changes in PR concentration, regulation and function in the myometrium throughout pregnancy and during labor. Quantification of PR in pregnant intrauterine tissues is difficult, in part due to a very low abundance of PR at the mRNA and protein level. The low abundance of the PR transcript was overcome in the present study using poly-adenylated RNA which provided a signal for PR well above the detectable sensitivity (Fig. 2). The lack of a fall in either peripheral plasma progesterone or myometrial PR mRNA speaks against a simple view of progesterone withdrawal as a major factor in the events leading to parturition in the baboon. In sheep, there is a fall in progesterone prior to labor but no similar change occurs in primates (Liggins

et al. 1973); however, replacement of progesterone to late-gestation physiological levels cannot arrest the initiation of labor in sheep (Liggins *et al.* 1973). Therefore, the precise function(s) of progesterone, in particular, throughout pregnancy and in the preparation for and completion of parturition, has not yet been defined in any species.

Estrogen and progesterone have no documented direct effect on contractility, but regulate contractility through their actions on protein synthesis, on synthesis of intracellular and cell surface receptors, on phospholipid membrane structure, and on eicosanoid synthesis (Garfield 1979). The polarity of the distribution of ER α and PR in the pregnant uterus is not required for mediating estrogen and progesterone physiological functions. Therefore, it is not surprising that there was no regional difference of either ER α or PR from the uterine fundus to the lower uterine segment.

In contrast to our observation with the steroid receptors, the labor-related elevation of OTR mRNA abundance in the fundus compared with the lower uterine segment suggests that OT exerts polarity in its effects on myometrial contractile force. This observation is consistent

Figure 5 (A) Northern analysis of TSP1 mRNA in non-labor (NL) lower uterine segment (LUS; lanes 1–9) and NL fundus (FUN; lanes 15–23) compared with spontaneous labor (SL) LUS (lanes 10–14) and SL FUN (lanes 24–28). (B) The same blot was probed for β -actin. (C) Densitometric analysis of the ratio of TSP1 and β -actin mRNA in NL LUS and NL FUN (means $\pm$ S.E.M.; $n=9$ for both groups) compared with SL LUS and SL FUN (means $\pm$ S.E.M.; $n=5$ for both groups). TSP1 mRNA increased significantly in both LUS and FUN during SL (* $P<0.05$). In addition, SL LUS TSP1 mRNA was significantly higher than SL FUN TSP1 mRNA (+ $P<0.05$).

with one previous report which used the binding activity measurement of a single human hysterectomy specimen, demonstrating that the increase in OTR numbers near term results in a gradient of expression, with the highest and lowest receptor concentrations found in the fundus and cervix respectively (Fuchs *et al.* 1984). This differential expression of OTR may be involved in directing the correct propagation of contractions from the fundus to the cervix during labor. We have recently reported a reduced contractile response of myometrium from the lower uterine segment tissues of the late-pregnant baboon to prostaglandin E2 which is paralleled by greater expression of inhibitory prostaglandin E2 receptor EP2 subtype,

and less contractile EP3 subtype expression (Smith *et al.* 1998). These data from our laboratory and others (Fuchs *et al.* 1984, Adenatado *et al.* 1988) raise the possibility that the expressions of mCAPs are not only under mechanical or hormonal control, but are also spatially regulated.

Myometrial sensitivity to OT is enhanced dramatically at term and during parturition due, at least in part, to an increase in the concentration of OTR (Fuchs *et al.* 1983). Data from the rat (Ou *et al.* 1998) and sheep (Wu *et al.* 1999b) demonstrate increased OTR expression during labor in the stretched gravid horn compared with the unstretched non-gravid horn in unilaterally pregnant animals. These findings suggest that mechanical distension

may be critical in regulating OTR mRNA levels during pregnancy and parturition. They are also compatible with stimulation of increased myometrial OTR mRNA by the increased uterine volume resulting from fetal growth as observed in the current study.

Our previous study in sheep (Wu *et al.* 1999a) and the present study, together with one previous report in pregnant women (Morimoto *et al.* 1998), indicate that increased myometrial TSP1 during labor is a common feature shared across species. The function of TSP1 in association with pregnancy and labor remains to be defined in relation to parturition. Both endogenous and exogenous TSP1 block blood vessel formation during physiological and pathological responses in which angiogenesis is a predominant feature (Good *et al.* 1990, Iruela-Arispe *et al.* 1991). Thus, increased myometrial TSP1 during labor may play a role in prevention of postpartum hemorrhage.

In summary, this is the first study to provide data in relation to regional distribution, gestation and labor on changes in mRNA for critical steroid receptor, peptide receptor and matrix protein mCAPs in the same animals in an extensively studied non-human primate. It is important that these changes were correlated to the status of myometrial contractility as monitored precisely and directly. There were differential changes of different mCAPs in relation to uterine topology, gestation and labor. The increased OTR mRNA associated with gestation may contribute to the increased uterine sensitivity to OT that occurs in preparation for labor. The higher OTR mRNA in the fundus compared with the lower uterine segment may be one of the mechanisms that provide polarity to the contraction of the uterus from the fundus to the lower uterine segment during labor. The observed increase of TSP1 mRNA in pregnant baboon myometrium during labor parallels findings in pregnant sheep and merits further study of its function in parturition. ER α and PR mRNA in the fundus and lower uterine segment did not change through the period of gestation studied or during spontaneous labor.

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