Characterization of the concentration gradient of prostaglandin H synthase 2 mRNA throughout the pregnant baboon uterus

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Abstract

The present study was designed to determine the effect of the spatial gradient from the cervix to the uterine fundus on the control of local prostaglandin H synthase (PGHS) 2 mRNA expression. We performed total cesarean hysterectomies during the last trimester in 12 pregnant baboons, 7 not in labor and 5 in labor, and examined PGHS2 mRNA expression throughout the uterus. PGHS2 mRNA abundance was quantified by *in situ* hybridization and northern blot analysis in the uterine fundus, lower uterine segment and the different segments of the cervix. Quantitative northern blot and *in situ* analysis demonstrated a gradient of PGHS2 mRNA expression, with the highest levels at the level of the lower portion of the cervix and decreased expression through the mid- and upper portion of the cervix and lower uterine segment; the lowest levels of expression were seen in the uterine fundus.

Moreover, cellular localization of PGHS2 mRNA and protein demonstrated high levels of expression in the cervical glandular epithelial cells with only occasional staining of smooth muscle cells in pregnant baboons. Decreased PGHS2 mRNA concentration gradient from the cervical external os to the fundus suggests that prostaglandin (PG) production in the uterus and cervix strongly depends on anatomical relations. This increased local PG production activity may be critical to pregnancy-associated lower uterine segment elongation, cervical softening and effacement in primate labor. These data provide a compelling biological basis for the use of PGHS2 inhibitors in the prophylaxis of preterm birth and cervical incompetence.

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Introduction

It has been 10 years since Drs MacDonald and Casey reported that levels of prostaglandins (PGs) in amniotic forebag were ten times higher than levels of PGs in the hindbag in pregnant women (MacDonald & Casey 1993). However, three fundamental questions relating to these high concentrations of forebag PGs remain unanswered: (1) What is their source (fetus or mother)? (2) What is their physiological significance? (3) What regulatory mechanisms control their production?

The amniotic forebag is the small amniotic sac that exists below the fetal presenting part, usually the fetal head. In late gestation the fetal presenting part engages, which divides the amniotic sac into two compartments: the upper compartment or hindbag and the lower compartment or forebag. Engagement of the presenting part also separates the amniotic fluid between upper and lower compartments. Myometrial contractures, which we and others have shown to be present throughout gestation in several

species (Nathanielsz *et al.* 1997), exert pressure through the amniotic forebag on the cervix and lower uterine segment and may constitute a significant force for producing prelabor regional anatomical changes. Myometrial contractures are characterized by non-painful discomfort, lack of intensity and a long duration compared with contractions.

However, myometrial contractures are more common and more intense near the end of pregnancy as the uterus prepares for labor. The amniotic forebag acts as a 'wedge' to efface the cervix before labor, driven by the force of contracture, and to dilate the cervix during labor, driven by the force of contraction. However, no study has addressed, in biochemical terms, the physiological significance of these remarkable prelabor regional changes in the lower uterine segment and the cervix in primate pregnancy. This shortcoming is in part due to the fact that the required tissues, i.e. cervix and lower segment, cannot be obtained from pregnant women.

In the last few years we have conducted a series of studies in the pregnant baboon from which we were able to obtain the required tissues and to address the biochemical mechanisms that underlie the high forebag PGs. We reported that the lower uterine segment expressed five to ten times higher prostaglandin H synthase 2 (PGHS2), the rate-limiting enzyme of PG synthesis, than the fundus in the pregnant baboon uterus in late gestation (Wu *et al.* 2000). Additionally, lower uterine segment and cervix, but not fundal PGHS2 increases with gestation and remains at high levels during spontaneous term labor. Our data suggested that the higher forebag levels of PGs levels are produced and accumulated locally.

This increased local PG production activity, reflected by higher forebag PG levels, may underlie the biochemical mechanisms associated with the dramatic anatomical changes of the lower uterine segment and the cervix that occur before the onset of labor.

In his classic paper of 20 years ago, Liggins *et al.* (1997) stated: 'We are almost completely ignorant of the physiological control of the pregnant cervix ... Cervical softening in prelabor must be explained in biochemical terms'. Cervical ripening remains a puzzle in primate labor. Endocervical application of exogenous synthetic PGE2 is an effective way of softening and preparing the cervix, as well inducing labor-type myometrial contractions and PGs can induce labor at any stage of human pregnancy (Husslein 1990). These findings indicate that high PG levels in the lower pole of the amniotic cavity may play a similar role in the prelabor cervical biochemical changes and preparation for labor *in vivo*. However, the cellular mechanisms controlling the high concentration of PGHS2 in the lower uterine segment and cervix, and producing the high concentration of PGs in the lower pole of the amniotic cavity, are poorly understood. The present study was designed to address: (1) the cellular distribution of PGHS2 mRNA throughout the uterus; (2) the effect of the spatial gradient from the uterine fundus to the cervix on the control of local PGHS2 mRNA expression; (3) the effect of gestation and labor on the control of uterine PGHS2 mRNA expression.

Materials and Methods

Animals

Estrous cycles were carefully followed in baboons maintained in social groups at the Southwest Foundation for Biomedical Research (San Antonio, TX, USA). Gestational age predicted from the cycles was confirmed by ultrasonography. Cesarean section hysterectomy under halothane general anesthesia was performed in seven baboons not in labor at: 121, 146, 153, 157, 177, 177 and 180 days gestation (dGA; term 175–185 dGA). Uterine electromyographic (EMG) leads had been previously sited in the baboons that underwent cesarean section hysterectomy

at 177, 177 and 180 dGA since it was particularly important to obtain myometrial activity recordings to ascertain that these animals were not in labor. Myometrial activity was monitored continuously and animals that were not in labor showed only low-amplitude, infrequent contractions. The cervix was uneffaced and closed in all of these animals. EMG analysis for 48 h preceding cesarean section hysterectomy revealed no contraction activity. Cesarean hysterectomy was also performed on five animals in spontaneous labor when myometrial activity was in the form of high-amplitude short-lived contractions. Four of these animals had myometrial EMG leads sited. The gestational ages at hysterectomy were 164, 184, 191 and 193 dGA. Cervical dilation was occurring in all of these animals and had reached 6, 3, 3 and 2 cm respectively. In a fifth animal without EMG electrodes, cesarean section hysterectomy was performed at 172 dGA with the cervix 4 cm dilated and fully effaced. Procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

Samples of cervix, lower uterine segment and fundus were dissected immediately and flash frozen for later RNA extraction or slowly frozen in dry-ice-cooled isopentane for *in situ* hybridization and immunocytochemistry analysis. The lower uterine segment was defined as the portion of the uterus superior to the internal cervical os and below a line 1 cm superior to the internal cervical os. The fundus was defined as the uterine portion immediately superior to the uterine cavity upper limit. The cervix was then evenly divided into three portions: internal os, mid-zone and external os. Each portion of the cervix was processed separately for later analysis.

Northern blot analysis

Polyadenylated RNA was extracted from frozen cervix, lower uterine segment and fundus by oligo-dT cellulose affinity chromatography using a commercial kit (Fast Track 2.0, Invitrogen). Northern blot analysis was performed as described previously (Wu *et al.* 2000).

In situ hybridization

Frozen sections (4-mm thick) cut onto commercially prepared poly-L-lysine-coated slides (Sigma) were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (20 min), washed twice in 0.1 M phosphate buffer, immersed in triethanolamine (TEA)-HCl (3.71 g TEA, 2 ml 6 M NaOH and 198 ml water), pH 8.0 and then TEA and acetic anhydride (0.25%) for 10 min. They were then washed in 2 × SSC for 5 min and briefly in 70% ethanol and allowed to air dry. The specimens were incubated for 2 h in a humidified container (55 °C) with 70 ml pre-hybridization buffer (50% freshly deionized

formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM sodium phosphate buffer (pH 8.0), 1 × Denhardt's solution), and for at least 16 h in hybridization buffer (i.e. pre-hybridization buffer plus probe = 1×10^6 c.p.m. per specimen in 70 ml). Control slides were hybridized in the presence of an excess of unlabelled antisense RNA. Slides were then washed: three times in 4 × SSC and 4 mM dithiothreitol (DTT; Sigma); three times in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl and 5 mM EDTA, pH 8.0) at 37 °C (second NTE wash was for 30 min with 30 mg/ml ribonuclease A (Sigma); 2 × SSC and 1 mM DTT; 0.1 × SSC and 1 mM DTT at 60 °C; and finally 0.1 × SSC at room temperature. The slides were then dehydrated in a graded series of ethanol plus 0.3 M ammonium acetate. They were air dried and exposed to autographic films for 2–4 days and then dipped in emulsion (Kodak NTB2) and exposed for 1–4 weeks at 4 °C. Following developing and fixing they were counterstained with hematoxylin and eosin, mounted and covered with a glass coverslip.

Image acquisition, processing and analysis

In situ autoradiograph hybridization signals of PGHS2 mRNA in the different segments of the uterus and the cervix were quantified by scanning densitometry. The relative optical density of the signal on autoradiographic film was quantified after subtraction of each individual background using a computerized image analysis system. The value obtained represents an average density over the area measured. Comparison between groups was performed using values obtained from three sections per animal.

Synthesis of probes

The cloned rhesus monkey PGHS2 cDNA (accession number, AF215734) in pCR II vector (Invitrogen), which include promoters for phage polymerases SP-6 to produce antisense probe and T-7 to produce sense probe, was linearized by an appropriate restriction enzyme and antisense and sense riboprobes were synthesized using a commercial kit (MAXIscript, Ambion, TX, USA) labeled with [α -32P] UTP for northern blot analysis or [α -35S] UTP for *in situ* hybridization (NEN Life Science, Boston, MA, USA).

Immunocytochemistry

Frozen sections (4 mm) of the pregnant baboon cervix, lower uterine segment and fundus were immunostained for PGHS2 protein using the avidin-biotin immunoperoxidase method as described previously (Wu *et al.* 1997). Specificity of immunostaining for the PGHS2 protein was

confirmed by two approaches: (1) omission of the primary antibody; (2) incubation of the slides with the normal goat serum instead of the primary antibody.

Statistical analysis

Since there were no differences in PGHS2 mRNA levels between term and spontaneous labor animals, data were combined in these animals. Comparison between groups was made by ANOVA and multiple *post-hoc* comparisons with Tukey's method for 95% confidence interval of pairwise differences. Statistical significance was assumed at the 5% level. Data are presented throughout as means $\pm$ S.E.M.

Results

Localization of PGHS2 mRNA and protein in the uterus

PGHS2 mRNA was mainly localized in glandular epithelial cells in both the cervix and the lower uterine segment (Fig. 1). Few smooth muscle cells in each segment of the uterus were positively stained for PGHS2 mRNA. Positive immunostaining for PGHS2 protein was also confined to the epithelial cells of the cervix and the lower uterine segment (Fig. 2) consistent with localization of PGHS2 mRNA. The staining for PGHS2 mRNA and protein was more abundant in the cervix or the lower uterine segment collected from pregnant baboons at term or during labor (Figs 1 and 2). No staining for PGHS2 mRNA and protein was observed in the fundus and no staining was observed when the PGHS2 antisense probe or primary antibody was replaced by sense probe or the normal goat serum.

Graded decrease of PGHS2 mRNA from the cervix to the fundus

Autoradiographic (Fig. 3) *in situ* analysis of PGHS2 mRNA showed a graded decrease of PGHS2 mRNA from the cervix to the fundus. This graded change was only observed in the uterus at term or during spontaneous labor (Fig. 3). The findings obtained by northern blot analysis were consistent with *in situ* hybridization analysis, i.e. there was a graded decrease of PGHS2 mRNA from the lower, middle and upper sections of the cervix, to the lower uterine segment and then to the fundus (Figs 3 and 4) at term or during spontaneous labor. PGHS2 mRNA abundance was highest in the lower and middle sections of the cervix (Figs 3 and 4).

The effect of gestation and labor on uterine PGHS2 mRNA expression

PGHS2 mRNA increased in late gestation and reached maximal levels around 170 dGA (Figs 3 and 4). There was

Figure 1 *In situ* microscopic analysis of cellular distribution of PGHS2 mRNA in fundus (FUN; A and D), lower uterine segment (LUS; B and E) and cervix (CX; C and F) of the pregnant baboons at 121 dGA (A, B and C) and at 180 dGA (D, E and F). Magnification: $\times 200$.

no further increase of PGHS2 mRNA in the lower uterine segment or the cervix associated with onset of spontaneous labor compared with term, as analyzed by *in situ* or northern blot analysis (Figs 3 and 4). There was no

difference in PGHS2 mRNA concentration between animals at different stages of cervical dilation. PGHS2 mRNA remained undetectable in the fundus during late gestation and labor.

Figure 2 Immunolocalization of PGHS2 protein in fundus (FUN; A and D), lower uterine segment (LUS; B and E), cervix (CX; C and F) of the pregnant baboons at 121 dGA (A, B and C) and at 180 dGA (D, E and F). Magnification: $\times 400$.

Figure 3 Representative autoradiographic *in situ* hybridization analysis of PGHS2 mRNA in external cervix (CXE), mid-zone cervix (CXM), internal cervix (CXI), lower uterine segment (LUS) and fundus (FUN) of (A) a pregnant baboon at 121 dGA not in labor and (B) a pregnant baboon at 193 dGA during spontaneous labor. (C) Densitometric analysis of autoradiographic *in situ* hybridization of PGHS2 mRNA signals in CXE, CXM, CXI, LUS and FUN of pregnant baboon at gestation <157 dGA ($n=4$, open bars) and at term or during spontaneous labor ($n=8$, solid bars). There was a graded decrease of PGHS2 mRNA from CXE to FUN at term and during SL. *, ** and +: $P<0.05$ compared with groups with different or no letters.

Figure 4 (A) Representative northern blot analysis of PGHS2 mRNA in external cervix (CXE; lanes 1–6), mid-zone cervix (CXM; lanes 7–12), internal cervix (CXI; lanes 13–18) and lower uterine segment (LUS; lanes 19–24) from the pregnant baboons at 121 dGA (lanes 1, 7, 13, 19), 157 dGA (lanes 2, 8, 14, 20) and 177 dGA (lanes 3 and 4, 9 and 10, 15 and 16, 21 and 22) and during spontaneous labor (lanes 5 and 6, 11 and 12, 17 and 18, 23 and 24). (B) β-Actin mRNA was used to control RNA loading. (C) Densitometric analysis of PGHS2 and β-actin mRNA ratio in CXE, CXM, CXI and LUS from the pregnant baboons at 121–157 dGA ($n=4$, open bars) and 177–180 dGA or during spontaneous labor ($n=8$, solid bars). * and +: $P<0.05$ compared with groups with different or no letters.

Discussion

The junction between the cervix and the uterine body is the internal os, and at this point the nature of the lining epithelium and uterine wall changes. The endocervical canal runs between the uterine and vaginal cavities and is lined by a single layer of tall columnar mucus-secreting epithelium that extends into the underlying stroma (endocervical mucus glands). These glandular structures are in fact deep slit-like invaginations of the surface epithelium, with blind-ended tubules (Stevens & Lowe 1992). Thus, there is a large surface area for the production

of cervical mucus, which fills the endocervical canal. We also observed a similar glandular structure in the lower uterine segment of the pregnant baboon uterus, which might result from pulling up of cervical tissues in late gestation. To our surprise it was this glandular structure that stained intensively for PGHS2 mRNA and protein. In contrast, cervical smooth muscle cells had almost negligible staining for PGHS2 mRNA and/or protein. For years, studies on cervical ripening have almost exclusively concentrated on the cervical connective tissues (Granstrom *et al.* 1989, Poma 1999) and smooth muscle cells (Poma 1999). Very little attention has been paid to the

function of the cervical glandular cells in the process of cervical ripening.

By secreting mucus, cervical glandular cells can provide a high-PG environment within the entire lower birth canal. Indeed, the quantities of PGE₂ and PGF₂ α recovered from vaginal fluid (by lavage) are higher during labor than before labor (Cox *et al.* 1993). This localized high-PG environment will be able to induce all the necessary changes that must occur in the lower birth canal for the proper completion of labor. These changes include lower uterine segment elongation, engagement of the fetal presenting part and cervical effacement. The engaged fetal presenting part (usually the fetal head) may act as a barrier to diffusion of forebag prostaglandins to the hindbag. It is critical that these important preparations occur without stimulating more generalized PG-regulated effects, such as myometrial stimulation throughout the uterus, during the critical preparturient period. Overstimulation of myometrial contraction at this time would be counter-productive.

This study is the first to report that expression of PGHS2 mRNA is strongly associated with the topographic structure of the primate uterus. Our data clearly showed that there was a significant decrease in PGHS2 mRNA concentration from the cervix to the fundus suggesting that the formation of a PG gradient throughout the uterus is a physiological event in late gestation and at labor (Romero *et al.* 1994). This increased local PG production activity may be critical for pregnancy-associated lower uterine segment elongation, cervical softening and effacement in primate labor. The increased lower uterine segment and cervical PGHS2 mRNA are probably regulated by both local and systemic factors. Stretch is a potential local factor recently shown to have powerful interactions with hormones in pregnant rat and sheep studies (Ou *et al.* 1998, Wu *et al.* 1999). We speculate that local stretch by the growing fetal presenting part, mediated through the amniotic forebag, may play a major role in stimulating lower uterine segment and cervical PGHS2 mRNA expression. Stretch produced as a consequence of fetal growth is a mechanical signal of fetal physical maturity sent to the mother.

In addition to the longitudinal gradient changes of PGHS2 mRNA expression throughout the uterus, we also observed a horizontal gestational-age-related change in PGHS2 mRNA expression. PGHS2 mRNA increased with gestation in the lower uterine segment and the cervix, indicating that other factors together with the local factors have synergistic effects in regulating cervical PGHS2 mRNA expression prior to labor. Estrogen is a potential candidate. We have previously shown that estradiol induced myometrial PGHS2 mRNA and protein expression in the pregnant sheep (Wu *et al.* 1997). Maternal plasma estrogens gradually increase throughout gestation and further increase at least 10 days before normal term labor in baboons (Wilson *et al.* 1991) and proportionately even earlier in human pregnancy (Buster

et al. 1979). We have also shown that increasing estrogen production by the infusion of androgen precursors produces vaginal delivery of live young in the rhesus monkey (Mecenas *et al.* 1996) and that this does not occur when conversion of androgen to estrogen is blocked by an aromatase inhibitor (Nathanielsz *et al.* 1998). Taken together these data indicate that estrogen is a major endocrine signal of fetal maturity and readiness. Circulating endocrine factors may play a role by interacting with local agents. Therefore the increase in PG concentration in the amniotic forebag with gestation is indirectly regulated by fetal endocrine maturity (estrogen) and physical readiness for birth (fetal growth).

Cervical ripening occurs in two steps: a slow stage extending over the major part of pregnancy and a final rapid process just preceding labor. We propose that PGs could induce both steps in cervical ripening, since PGs can induce labor at any gestational stage of human pregnancy.

The high level of PGs in the forebag is very likely to be the major mechanism for the rapid process of cervical preparation near term. By containing a high concentration of PGs near term the amniotic forebag acts not only as a mechanic 'wedge', but also moves as an *in vivo* PG-filled water ball to efface the softened cervix under the force of myometrial contractures. Such a scenario would produce all the clinical signs occurring in very late gestation, such as fetal presentation engagement, cervical effacement and 'clinical show'. The combinations of the mechanical (hydrostatic pressure applied from the amniotic forebag) and biochemical (PGs accumulated in the amniotic forebag) forces rapidly prepares the cervix and lower birth canal in late gestation when the fetus is ready for delivery.

The lower PG concentrations in the hindbag (MacDonald & Casey 1993) probably reflect the failure of fundal PGHS2 production to increase before labor, as we reported previously (Wu *et al.* 2000). In addition, engagement of the presenting part will act as a barrier to diffusion of forebag PGs to the hindbag. Then how can fundus and the rest of the uterus contract strongly during labor? Several mechanisms underlie increased PG concentrations in the hindbag to stimulate the strong contraction of the entire uterus at labor when the low uterine segment and the cervix are fully prepared. Our earlier data indicated a labor-related increase in amnion PGHS2 mRNA (Wu *et al.* 2000). In addition, the lower uterine segment and the effaced cervix are drawn up towards the hindbag. Furthermore, we have shown a stronger contractile response to PGE₂ of pregnant baboon myometrial strips from the fundus compared with the lower uterine segment, which was related to higher expression of the gene encoding the contractile EP₃ receptor and lower expression of the gene encoding the inhibitory EP₂ receptor in fundal myometrium compared with the lower uterine segment (Gordon *et al.* 1998). Together, all of these synergistic mechanisms will make the uterus contract

when the lower birth canal is prepared and will bring labor to a conclusion.

Conclusions

PGHS2 mRNA was mainly localized in glandular epithelial cells of the cervix and the lower uterine segment in pregnant baboons. A decreasing PGHS2 concentration gradient from the cervix to the fundus suggests that PG production in the uterus strongly depends on anatomical relations. This increased local PG production activity may be critical to pregnancy-associated lower uterine segment elongation, cervical softening and effacement in primate labor.

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