Localization and steroid regulation of prostaglandin E₂ receptor protein expression in ovine cervix

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Abstract

Although prostaglandin E₂ (PGE₂) has been identified as a central mediator of the cervical ripening process, the mechanisms responsible for PGE₂ ripening are still poorly understood, partly because of the lack of information concerning the precise cellular localization and regulation of PGE₂ (EP) receptors in the cervix. To provide new insights into the mechanisms of cervical ripening, we used indirect immunofluorescence to localize cervical EP receptor protein expression in ovariectomized ewes and examined the effect of administration of progesterone or estradiol. EP receptors were widely distributed in cervical blood vessels, epithelium of the cervical canal, circular and longitudinal muscles, and stroma. Estradiol replacement decreased EP₁ and EP₃ receptor protein in blood vessel media (by 23 and 31% respectively, $P < 0.05$) and decreased EP₁ receptor protein expression in the longitudinal muscle layer (by 27%, $P < 0.05$). Stromal EP₁ and EP₃ receptor protein expression was also reduced by estradiol (by 29 and 20% respectively, $P < 0.05$). Progesterone replacement had no significant effect on EP receptor protein expression. The arterial changes would favor PGE₂-induced vasodilatation, subsequent edema and leukocyte infiltration during the cervical ripening process whereas the muscular alterations would facilitate smooth muscle relaxation and cervical dilatation. Furthermore, estradiol provoked perinuclear localization of EP₃ receptor protein in the longitudinal muscle layer. This latter result suggests that cellular EP receptor localization is regulated by estradiol and that PGE₂ may also control smooth muscle contraction and regulate ovine cervical dilatation in an intracrine manner via EP₃ receptors.

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Introduction

Cervical ripening is an active process, independent of uterine contraction (Stys *et al.* 1978), which ensures that the cervix becomes a soft and compliant tissue able to dilate to permit the passage of the fetus during labor. Softening of the cervix involves a complex combination of biochemical and structural changes affecting the cervical stroma, and leading to an extensible organ (Uldbjerg *et al.* 1983). Human cervical ripening is characterized by: edema (Leppert 1995); leukocyte infiltration (Junqueira *et al.* 1980); dispersion of the collagen network (Danforth *et al.* 1974) mainly resulting from collagen degradation by leukocyte-released matrix metalloproteinases; and an increase in total glycosaminoglycans (GAGs) (Osmers *et al.* 1993). Similar changes are observed in sheep cervix (Fosang *et al.* 1984, Fosang & Handley 1988).

Several lines of evidence support a pivotal role for prostaglandin E₂ (PGE₂) in the regulation of the cervical ripening process. First, cyclooxygenase-2 (COX-2) expression

(Dong *et al.* 1996) and PGE₂ (Mitchell & Flint 1977) synthesis increase at the time of parturition in the rat and sheep cervix. Secondly, treatment with COX-2 inhibitors delays this process (Mitchell & Flint 1978). Finally, local application of PGE₂ has been used clinically for 25 years (Hughes *et al.* 2001) to induce softening of the cervix successfully at term human pregnancy. The changes in the composition of the cervical connective tissue after PGE₂-induced cervical ripening are similar to those occurring in spontaneous cervical ripening (Danforth *et al.* 1974, Junqueira *et al.* 1980, Cabrol *et al.* 1987, Allen *et al.* 1988, Osmers *et al.* 1991, 1993, Leppert 1995, Kelly 2002). Indeed, PGE₂ increases human cervical collagenolytic activity (Osmers *et al.* 1991) and GAG synthesis in rat cervix (Cabrol *et al.* 1987), induces vasodilatation of human cervical arteries (Allen *et al.* 1988) and thus promotes subsequent edema and leukocyte infiltration (Kelly 2002).

PGE₂ transduces its signal via seven-transmembrane domain, G protein-coupled receptors, called EP receptors

(Kennedy *et al.* 1982, Narumiya *et al.* 1999). Ligand-binding studies have demonstrated the presence of these receptors in pregnant human cervical tissues (Adelantado *et al.* 1988). The EP receptor family has been further classified into four subtypes (EP₁, EP₂, EP₃ and EP₄ (Narumiya *et al.* 1999)), differing in their structure, ligand-binding affinities and signal transduction pathways (Narumiya *et al.* 1999). EP₁ and EP₃ receptors cause smooth muscle contraction, while EP₂ and EP₄ induce relaxation of smooth muscle (Narumiya *et al.* 1999).

Even though it has been demonstrated that PGE₂ increases GAG synthesis via EP₄ receptors in human cervical fibroblasts (Schmitz *et al.* 2001, 2003), the mechanisms responsible for PGE₂ ripening effects are still poorly understood – partly because of the lack of information concerning the localization and the regulation of EP receptors in the cervix. Up-regulation of contractile EP₃ and/or down-regulation of relaxant EP₂ receptor mRNA have been reported in the myometrium of pregnant rats (Brodth-Eppley & Myatt 1998), ewes (Ma *et al.* 1999), baboons (Smith *et al.* 2001a) and women (Brodth-Eppley & Myatt 1999) at parturition. These EP receptor labor-associated alterations suggest that EP receptor expression is hormonally regulated at the time of labor, by the changes in progesterone and estrogens associated with parturition (Challis 1971, Buster *et al.* 1979, Romero *et al.* 1988, Challis *et al.* 2000).

In contrast to the considerable literature concerning EP receptor function, expression and regulation in myometrium, there is little information on key issues such as cellular distribution of EP receptor expression and regulation in the uterine cervix. The purpose of this study was to localize the four EP receptor proteins for the first time to specific cell types in the cervix of ovariectomized ewes and to examine their regulation by progesterone and estradiol, in order to provide new insights into the hormonal mechanisms controlling cervical ripening.

Materials and Methods

Animals and tissue collection

Surgical procedures were performed at Cornell University (Ithaca, NY, USA) and were approved by the Cornell University Institutional Animal Care and Use Committee. The Cornell facilities are approved by the American Association for the Accreditation of Laboratory Animal Care. Eighteen non-pregnant ewes (*Ovis aries*) were ovariectomized on the day of ovulation, 48 h after removal of progesterone sponges used to synchronize estrus. After 10 days, as previously described (Wu *et al.* 1996, 1997, 2000, 2003), ewes received one of the following treatments: saline infusion controls ($n = 6$), estradiol (Sigma) infused intravenously for 2 days (50 µg/day in 0.5 ml saline per hour, $n = 6$), or an intravaginal progesterone sponge for 10 days (containing 0.3 g progesterone, $n = 6$). Progesterone sponges were purchased from Carter Holt

Harvey Plastic Products (Hamilton, New Zealand) and produced plasma concentrations in the range 1.5–2 ng/ml (provided by the manufacturer). All animals were infused with the same volume of physiological saline as the control group. Cervices were removed from the body of the uterus at necropsy under halothane general anesthesia, rinsed in physiological saline, fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA, USA) and embedded in paraffin. One piece of cervical tissue from one control animal was flash frozen to provide protein to validate the antibodies by Western blot analysis.

Western blot analysis

Western blots were run under denaturing and reducing conditions. Approximately 0.1 g of cervical tissue was homogenized in 1 ml RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 1% NP40, 150 mmol/l NaCl, 0.1% SDS, 0.25% sodium deoxycholate) supplemented with a cocktail of protease inhibitors. Samples were centrifuged at 12 000 g for 15 min to pellet cellular debris. Protein extracts were quantitated using a modified Bradford technique and BSA as a standard. Protein extracts (10 µg) were dissolved v/v in 2 × Tris-glycine SDS sample buffer (126 mmol/l Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue, pH 6.8) (Invitrogen) supplemented with 10 mmol/l dithiothreitol (DTT) and heated at 95 °C for 5 min before analysis on a 7.5% SDS-PAGE. After migration, proteins were transferred to a PVDF membrane (Amersham). Membranes were blocked in 10% non-fat dried milk powder in Tris-buffered saline-Tween 20 (TBS-T) (25 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween-20, pH 7.6) for 1 h. For immunodetection, blots were incubated with rabbit polyclonal antibodies against human EP receptors (Cayman Chemicals, Ann Arbor, MI, USA; catalog numbers 101740, 101750, 101760 and 101775) for 90 min at a dilution of 1:1000 in TBS-T containing 5% non-fat dried milk powder. After five washes in TBS-T, blots were incubated with horseradish peroxidase-linked goat anti-rabbit (SantaCruz Biotechnologies, Santa Cruz, CA, USA; catalog number sc-2004) for 45 min in TBS-T containing 5% non-fat dried milk powder at a dilution of 1:2000. Immunoreactive proteins were detected using a chemiluminescent detection system (Amersham ECL reagents, Amersham) according to the manufacturer's directions. Control experiments were performed by incubating the membranes with anti-human EP receptor antibodies pre-absorbed on the related blocking peptides at a dilution of 1:100 (Cayman Chemicals; catalog numbers 301740, 301750, 301760 and 301775).

Indirect immunofluorescence

Sections (5 µm thick) were cut from the paraffin-embedded tissues, mounted on ProbeOn Plus microscope slides (Fisher Scientific), deparaffinized in xylene and rehydrated in a graded ethanol series. Sections were

washed in deionized water, and antigen was retrieved by microwaving in a polypropylene Coplin staining jar (VWR International, Aurora, CO, USA) at full power for 10 min in DAKO target retrieval solution (pH 6.20) (DAKO Corporation, Carpinteria, CA, USA). All sections were washed in PBS then blocked for 30 min with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Sections were then incubated for 1 h at room temperature with the primary antibody diluted in DAKO antibody diluent with background reducing components (DAKO Corporation). Polyclonal primary antibodies were used at 1:250 dilutions. Sections were washed in PBS and then further incubated with the second antibody (biotinylated goat anti-rabbit IgG) (Vector Laboratories) diluted 1:200 in 2% goat serum in PBS for 30 min at room temperature. Sections were washed and the antigen was localized using 2.5 µg/ml fluorescein streptavidin (Vector Laboratories) in PBS for 15 min. Slides were extensively washed and then mounted with Vectashield hard-set mounting medium (Vector Laboratories). To control for variability in intensity of staining, slides from different groups were stained at the same time. Negative controls included slides incubated without the primary antibody and sections incubated with non-immune rabbit serum (Vector Laboratories) instead of primary antibody.

Image analysis

Fluorescence staining was observed using a Nikon TE300 inverted microscope (Nikon USA, Melville, NY, USA). Images were captured with a CoolSNAP HQ cooled charge-coupled device (CCD) digital camera (Roper Scientific, Trenton, NJ, USA) coupled to the computer image analysis software Metamorph (Universal Imaging Corporation, Downingtown, PA, USA). Evaluation of all slides was performed by an investigator who was blind to the animal treatment. For each of the five different tissue components studied (blood vessels, mucosa, longitudinal muscle layer, circular muscle layer and stroma), three different pictures corresponding at widely separated positions on the slide were taken at a magnification of $\times 100$ (15 pictures per slide). Quantification of EP receptor immunoreactivity was performed with ImageJ v1.31 (<http://rsb.info.nih.gov/ij/>) image analysis software. The average gray level of fluorescence immunoreactivity was measured on the whole surface of the picture (696 $\times$ 520 pixels) for the stroma. For the endothelium, the media, the epithelium of the cervical canal and the longitudinal and circular muscle layers, the whole surface of each tissue present in the picture was selected using the freehand selection tool and the average gray level of fluorescence immunoreactivity was measured in the selected areas.

Statistical analysis

The gray level of fluorescence immunoreactivity for each tissue component of each animal was taken as the mean

of three different measurements of the average gray level of fluorescence immunoreactivity (sum of the gray values of all the pixels in the selection divided by the number of pixels) from three different areas of the slide. The gray level of fluorescence immunoreactivity for each tissue component in each group was expressed as the mean $\pm$ S.E.M. of six different values from the different animals composing each group (i.e. control, progesterone and estradiol groups). The gray levels of fluorescence immunoreactivity were then expressed in simpler relative numerical values (1.30 or 0.21 rather than 1296 and 210) by dividing all the values by 1000.

Statistical comparisons between control, progesterone and estradiol groups were carried out using a one-way ANOVA. Differences in EP receptor expressions were determined by *post hoc* tests using the Bonferroni correction. The level of significance was set at $P < 0.05$.

Results

Specificity of rabbit polyclonal anti-human EP receptor antibodies in sheep cervix

The specificity of the different anti-human polyclonal antibodies for all four sheep EP receptor proteins was assayed by Western blot analysis. As shown in Fig. 1, distinct proteins of different apparent molecular masses were identified by the various rabbit anti-human EP receptor antibodies. We observed a protein band of approximately 56 kDa by staining with anti-EP₁ antibody, a 67 kDa band with the anti-EP₂ antibody, a 62 kDa band with the anti-EP₃ antibody and a 64 kDa band with the anti-EP₄ antibody. Furthermore, pre-absorbing the primary antibodies, with the antigen to which they were raised, abolished the 56, 67, 62 and 64 kD signals (Fig. 1).

Cervical distribution of EP receptor proteins

Indirect immunofluorescence demonstrated the expression of all four EP receptor proteins in ewe cervixes. EP receptor localization is shown in Fig. 2. The EP₁, EP₂, EP₃ and

Figure 1 Specificity of rabbit polyclonal anti-human EP receptor antibodies in sheep cervix. EP receptor expression in sheep cervix was assayed by Western blot analysis as described in the Materials and Methods. Western blots demonstrated only a single band. Pre-absorption of the primary antibodies with the related blocking peptide (BP) ('-' signifies BP added) abolished the specific signals.

EP₄ receptor proteins were expressed in all six different tissue components studied – i.e. the endothelium and smooth muscle of cervical blood vessels, the epithelium of the cervical canal, the circular and longitudinal muscle

layers, and the stroma. The absence of detectable immunoreactive protein when the primary antibody was replaced by non-immune rabbit serum confirmed the specificity of the staining.

Figure 2 EP receptor immunolocalization in cervical tissues of control ewes. EP₁ (A, E, I, M, Q), EP₂ (B, F, J, N, R), EP₃ (C, G, K, O, S) and EP₄ (D, H, L, P, T) receptor localization was assayed by indirect immunofluorescence as described in the Materials and Methods. Receptors were localized in blood vessels (A, B, C, D), epithelium of the cervical canal (E, F, G, H), circular muscle layer (I, J, K, L), longitudinal muscle layer (M, N, O, P) and stroma (Q, R, S, T). Negative controls were performed by replacing the first antibody by non-immune rabbit serum (U).

Steroid regulation of cervical EP receptor protein expression

Progesterone replacement had no significant effect on the distribution of the four EP receptor proteins in the six tissue components compared with the control group (Table 1). Compared with the control group, the expression of EP₁ receptor protein in the media, the longitudinal muscle layer and in the stroma was significantly reduced in the estradiol-replaced group ($P < 0.05$) (Table 1). The expression of EP₃ receptor protein, in the media and in the stroma of the estradiol-replaced animals, was also significantly decreased compared with the control and progesterone-replaced groups ($P < 0.05$) (Table 1).

Estrogen-dependent alteration of EP₃ receptor cellular localization

Immunofluorescent staining demonstrated perinuclear localization of EP₃ receptor protein expression in the longitudinal muscle layer of all six estradiol-replaced ewes (Fig. 3), but not in any other tissue in this group.

Table 1 Effects of progesterone and estradiol replacement on cervical EP receptor protein expression.

Cervical tissues	Control (n = 6)	Progesterone (n = 6)	Estradiol (n = 6)
EP ₁ expression			
Endothelium	1.21 ± 0.10	1.26 ± 0.10	1.05 ± 0.07
Media	1.07 ± 0.06	1.03 ± 0.09	0.83 ± 0.05*
Epithelium	1.15 ± 0.11	1.15 ± 0.10	0.94 ± 0.07
Circular muscle layer	0.96 ± 0.09	0.97 ± 0.09	0.76 ± 0.09
Longitudinal muscle layer	1.27 ± 0.10	0.99 ± 0.08	0.88 ± 0.08*
Stroma	0.74 ± 0.07	0.68 ± 0.04	0.53 ± 0.05*
EP ₂ expression			
Endothelium	1.35 ± 0.17	1.45 ± 0.23	1.29 ± 0.18
Media	1.01 ± 0.10	0.99 ± 0.12	0.95 ± 0.15
Epithelium	1.31 ± 0.20	1.61 ± 0.19	1.26 ± 0.18
Circular muscle layer	0.87 ± 0.11	0.99 ± 0.11	0.77 ± 0.11
Longitudinal muscle layer	0.93 ± 0.12	1.13 ± 0.14	0.81 ± 0.11
Stroma	0.73 ± 0.09	0.83 ± 0.06	0.65 ± 0.08
EP ₃ expression			
Endothelium	1.39 ± 0.07	1.52 ± 0.13	1.27 ± 0.07†
Media	1.23 ± 0.09	1.11 ± 0.09	0.90 ± 0.06*
Epithelium	1.25 ± 0.14	1.31 ± 0.15	1.20 ± 0.10
Circular muscle layer	0.98 ± 0.06	1.20 ± 0.10	0.99 ± 0.05
Longitudinal muscle layer	1.15 ± 0.14	1.31 ± 0.15	1.16 ± 0.09
Stroma	0.80 ± 0.01	0.86 ± 0.08	0.64 ± 0.02*†
EP ₄ expression			
Endothelium	1.07 ± 0.09	1.10 ± 0.09	1.03 ± 0.10
Media	0.85 ± 0.08	0.98 ± 0.08	0.88 ± 0.08
Epithelium	1.11 ± 0.13	1.14 ± 0.11	0.93 ± 0.05
Circular muscle layer	0.82 ± 0.10	0.91 ± 0.06	0.74 ± 0.03
Longitudinal muscle layer	0.88 ± 0.09	0.87 ± 0.08	0.77 ± 0.03
Stroma	0.79 ± 0.08	0.68 ± 0.05	0.65 ± 0.02

Results are expressed as means ± S.E.M. of gray levels of fluorescence immunoreactivity. Statistical comparisons between groups were carried out using a one-way ANOVA. Differences in EP receptor expressions were determined by *post hoc* tests using the Bonferroni correction.

* $P < 0.05$ versus control.

† $P < 0.05$ versus progesterone.

Perinuclear localization of EP receptor proteins was not observed in the tissues of the control (Fig. 2) or progesterone-replaced ewes for any EP receptor subtype.

Discussion

Until recently, the lack of EP receptor antibodies has prevented any attempt to study the precise EP receptor cellular distribution at the protein level in tissues of the female reproductive tract. The recent development of EP receptor antibodies enables localization as well as examination of steroid regulation of EP receptor protein expression in the tissues that compose the cervix.

In the present study, we first checked the cross-reactivity of the available rabbit anti-human EP receptor antibodies with EP receptors in the sheep cervix by running Western blot analysis which demonstrated single bands for EP₁, EP₂, EP₃ and EP₄ receptors of approximately 56, 67, 62 and 64 kDa respectively. These values agree with the molecular mass range reported in human tissues (Morath *et al.* 1999) and rat (Southall & Vasko 2001). The specificity of the immunoreactive detection was further confirmed by the absence of signal when the primary antibodies were pre-absorbed by the original peptide antigen. Furthermore, the homology between the human and published ovine protein sequences, in the highly conserved C-terminal region against which the antibodies were designed, is high (85–90%), suggesting a specific antigen–antibody interaction.

We demonstrated the presence of all four EP receptor proteins, confirming previous data obtained at the RNA level in baboons (Smith *et al.* 2001b), in all the tissue components of the ovine cervix. During cervical ripening, PGE₂ is generally considered to act mainly as an inducer of stromal extracellular matrix protein and glycoprotein alteration. The localization of the four EP receptors in blood vessels emphasizes components of the vascular system as possible targets for PGE₂. PGE₂ exerts its effects on blood vessels through actions on multiple counter-balanced signaling pathways in vascular smooth muscle cells. EP₁ and EP₃ receptors induce vasoconstriction whereas EP₂ and EP₄ receptors provoke vasodilatation (Wright *et al.* 2001). It is not possible from our results to predict the overall effect that PGE₂ exerts on cervical blood vessels. However, the fact that EP receptors are expressed on both the endothelium and smooth muscle of cervical blood vessels suggests that PGE₂ can act as an important modulator of cervical vascular tone. Any modification of the contractile/relaxant EP receptor ratio will affect the ability of PGE₂ to provoke either vasoconstriction or vasodilatation.

In order to evaluate the effects of steroid hormones on EP receptor expression, ovariectomized ewes received replacement therapy with either progesterone or estradiol; we have used this model extensively in previous work (Wu *et al.* 1996, 1997, 2000, 2003). EP₁ and EP₃ receptor

Figure 3 Perinuclear expression of EP₃ receptors in estradiol-replaced ovariectomized ewes. EP receptor localization was assayed by indirect immunofluorescence as described in the Materials and Methods. Immunofluorescent staining demonstrated perinuclear localization (arrows) of EP₃ receptors only in the longitudinal muscle layer of estradiol-replaced ewes (B) and not in controls (A).

protein expression were both significantly decreased in the blood vessel media of estradiol-replaced ewes compared with control tissues (by 23 and 31% respectively). Estradiol concentrations rise in ovine maternal plasma at parturition (Challis 1971) and prostaglandins mediate premature delivery induced by estradiol in pregnant sheep and goats (Currie *et al.* 1976, Wu *et al.* 2004). Estradiol, by decreasing EP₁ and EP₃ receptor expression, would facilitate EP₂ and EP₄ receptor-dependent vasodilator effects of PGE₂ and thus promote cervical ripening. Interestingly, it has been demonstrated by competition binding studies and measurement of vascular resistance, in newborn versus adult porcine choroidal vasculature, that fewer EP₁ receptors (33 versus 55%) and more EP₂ receptors (33 versus 8%) were responsible for the increased vasodilatation in the newborn (Abran *et al.* 1997). Therefore, even modest variation in cervical EP receptor expression might be sufficient to provoke physiological alterations in the vascular response to PGE₂.

Both *in vitro* and *in vivo* studies suggest an important role for EP₄ receptor in mediating PGE₂ ripening effects. PGE₂ induces GAG synthesis by human cervical fibroblasts via EP₄ receptors (Schmitz *et al.* 2001, 2003), and EP₄ receptor agonists increase pregnant guinea-pig cervical compliance (Kanayama *et al.* 2004). Therefore, the significantly decreased expression of EP₁ and EP₃ receptors (by 29 and 20% respectively) in the stroma after estradiol replacement might play a role by altering the balance of positive and negative influences. As a result, the activation of EP₄ receptors by PGE₂ in this tissue compartment would represent a second mechanism for estradiol to promote cervical ripening.

Estradiol provoked a significant 27% decrease in EP₁ receptor protein expression in the longitudinal muscle layer of the estradiol-replaced animals in comparison with the control group. Hollingsworth and Isherwood (1978) demonstrated decreased contractile response of cervical strips to PGE₂ at the end of rat gestation. Decreased

expression of contractile EP receptor would contribute to a better dilatation of the cervix during parturition.

Progesterone replacement had no significant effect on EP receptor distribution in the ovine cervix. Conversely, a significant increase in EP₂ receptor mRNA expression in ovariectomized rat myometrium replaced by progesterone has been demonstrated (Dong & Yallampalli 2000). We have previously demonstrated different regulatory effects of progesterone on COX-2 protein expression in endometrium and myometrium using the ovine model (Wu *et al.* 1997). Similarly, progesterone regulation of EP receptor expression is likely to be tissue and/or species dependent. Differences in transcription and translation of the different receptors may also explain species- and tissue-specific changes.

In addition to the changes in EP₁ and EP₃ receptor tissue distribution, our results clearly demonstrate that estradiol alters cellular EP receptor localization. After estradiol replacement, indirect immunofluorescence demonstrated perinuclear expression only of EP₃ receptor protein in the longitudinal muscle layer. This localized distribution was not observed in the tissues from the control ewes. These findings are in accordance with recent observations demonstrating perinuclear localization of EP receptors in porcine cerebral microvascular endothelial cells (Bhattacharya *et al.* 1998, 1999), in fibroblast cell lines (Bhattacharya *et al.* 1998), in human embryonic kidney cells (Bhattacharya *et al.* 1999), in human ocular tissues (Schlotzer-Schrehardt *et al.* 2002), in human adult and fetal bone tissues (Fortier *et al.* 2004) and in human myometrium (Astle *et al.* 2005). To our knowledge, this is the first report of an estradiol-dependent regulation of perinuclear EP receptor expression. Activation of pig perinuclear EP receptors by PGE₂ modulates nuclear calcium transient and transcription of genes such as inducible nitric oxide synthase (NOS) (Bhattacharya *et al.* 1999) and endothelial NOS (Gobeil *et al.* 2002). Therefore, PGE₂ might increase NO synthesis and facilitate cervical

dilatation during parturition in an intracrine manner via the activation of perinuclear EP₃ receptor in the longitudinal muscle layer. Finally, estradiol-dependent expression of perinuclear EP₃ receptor might represent a novel indirect pathway for estradiol to regulate gene expression. These hypotheses merit study in *in vitro* paradigms.

In conclusion, EP receptors are widely distributed within cervical tissues and predominantly expressed in blood vessels. Estradiol replacement in the ovariectomized ewes decreased EP₁ and EP₃ receptor protein expression in the blood vessel media and decreased EP₁ receptor protein expression in the longitudinal muscle layer. These changes would favor PGE₂-induced vasodilatation, subsequent edema and leukocyte infiltration during the cervical ripening process as well as facilitating smooth muscle relaxation during cervical dilatation. Furthermore, estradiol administration results in perinuclear expression of the EP₃ receptor in the longitudinal muscle layer. This finding suggests that EP receptor locations are not only regulated by estradiol at the tissue level, but also at the cellular level, and that PGE₂ may control smooth muscle contraction and regulate ovine cervical dilatation in an intracrine manner via EP₃ receptors.

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