Cyclooxygenases and prostaglandin E synthases in the endometrium of the rhesus monkey during the menstrual cycle

Tong Sun¹, Shi-Jie Li¹, Hong-Lu Diao¹, Chun-Bo Teng¹, Hong-Bin Wang²
and Zeng-Ming Yang¹

Colleges of ¹Life Sciences and ²Animal Medicine, Northeast Agricultural University, Harbin 150030, China

Correspondence should be addressed to Zeng-Ming Yang; Email: zmyang@mail.neau.edu.cn

Abstract

Cyclooxygenase (COX), a rate-limiting enzyme that produces prostaglandins (PGs) from arachidonic acid, exists in two isoforms, COX-1 and COX-2. PGE₂ synthase (PGES) is a terminal prostanoid synthase and can enzymatically convert the cyclooxygenase product PGH₂ to PGE₂, including two isoforms: microsomal PGES (mPGES) and cytosolic PGES (cPGES). cPGES is predominantly linked with COX-1 to promote the immediate response. mPGES is preferentially coupled with the inducible COX-2 to promote delayed PGE₂ generation. COX-2-deficient female mice are infertile with abnormalities in ovulation, fertilization, implantation and decidualization. The aim of this study was to examine immunohistochemically the expression pattern of COX-1, COX-2, mPGES and cPGES proteins in the endometrium of the rhesus monkey during the menstrual cycle. COX-1 immunostaining was mainly localized in the luminal epithelium and glandular epithelium near the lumen, and detected in all the stages during the menstrual cycle. COX-2 immunostaining was mainly localized in the luminal and glandular epithelium, and strongly shown during the mid-luteal phase (days 16 and 20) of the menstrual cycle. There was a strong cPGES immunostaining in the luminal and glandular epithelium on days 12, 16, 20 and 25 of the menstrual cycle. mPGES immunostaining was strongly detected in the glandular epithelium on days 20 and 25 of the menstrual cycle. These data suggest that the coupling of cPGES and COX-1 in the luminal epithelium may be responsible for the synthesis of PGE₂ in monkey endometrium, and the coupling of mPGES and COX-2 in the glandular epithelium may be of importance for preparing the receptive endometrium.

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Introduction

Prostaglandins (PGs) are considered to be proinflammatory and to play important roles during female reproduction (Downie *et al.* 1974, Lim *et al.* 1997). Cyclooxygenase (COX) is a rate-limiting enzyme that produces PGs from arachidonic acid. There are two isoforms: COX-1 and COX-2. COX-1 appears to be constitutively synthesized in many tissues, although its level of expression can vary with the state of differentiation or following cytokine or tumor promoter stimulation (Seibert *et al.* 1994, Samet *et al.* 1995). However, COX-2 expression is upregulated in response to various proinflammatory or mitogenic agents (Hla & Neilson 1992, Smith *et al.* 1994). COX function in mice has been demonstrated with gene knockout experiments. COX-1-deficient females are fertile with specific parturition defects, whereas COX-2-deficient females are infertile with abnormalities in ovulation, fertilization, implantation and decidualization (Dinchuk *et al.* 1995, Langenbach *et al.* 1995, Lim *et al.* 1997). Recently, in another study, COX-2-deficient mice did not show any abnormality in embryo implantation except for the

reduction in the numbers of ovulated and fertilized eggs, and a delay in decidual growth (Cheng & Stewart 2003). What causes this difference is still not clear.

PGE₂ synthase (PGES) is a terminal prostanoid synthase that can enzymatically convert the cyclooxygenase product PGH₂ to PGE₂. There are two isoforms of PGES, microsomal PGES (mPGES) and cytosolic PGES (cPGES). cPGES is constitutively expressed in a wide variety of cells and tissues, and predominantly linked with COX-1 to promote the immediate response, during which relatively high concentrations of arachidonic acid are released in a short period (Tanioka *et al.* 2000). mPGES, a membrane-associated and inducible perinuclear enzyme with glutathione-dependent activity, is expressed in a variety of tissues, including prostate, testes, and small intestine (Jakobsson *et al.* 1999). mPGES is preferentially coupled with the inducible COX-2 to promote delayed PGE₂ generation and, if COX-2 already exists in cells, it also regulates immediate PGE₂ generation (Murakami *et al.* 2000).

The expression and regulation of the mPGES gene in mouse uterus was recently characterized (Ni *et al.* 2002). The strong mPGES expression in the implantation site and

decidual cells suggests that mPGES might play an important role during implantation and, more importantly, decidualization (Ni *et al.* 2002). Recently, it has been found that COX-2 mRNA and protein and mPGES mRNA are differentially expressed and regulated in bovine endometrium during the estrous cycle. The COX-2 and mPGES pathway is responsible for the endometrial production of PGE₂ in the bovine endometrium during the estrous cycle (Arosh *et al.* 2002). Expression of mPGE synthase was localized in glandular epithelial and endothelial cells in both basalis and functionalis regions of the human endometrium. By contrast, stromal staining was predominantly localized in the functionalis layer (Milne *et al.* 2001).

The expression of cPGES in the uterus was reported only in mice. There was a strong level of cPGES mRNA signal in the stromal cells at the implantation site on day 5 of pregnancy, while cPGES immunostaining was strongly detected in the luminal epithelium. The signals for both cPGES mRNA and immunostaining were strongly detected in the decidualized cells from days 6–8 of pregnancy. These data suggest that cPGES may play an important role during implantation and decidualization (Ni *et al.* 2003).

In spite of the extensive studies reported on COX expression, regulation and potential function in the reproductive processes of other species, relatively little is known on COX expression and regulation in the primate uterus. Kim *et al.* (1999) have reported the expression of COX-1 and COX-2 during the menstrual cycle and pregnancy in the baboon. In man, COX-2 was localized in the uterine glandular epithelium by immunohistochemistry throughout the menstrual cycle and in early pregnancy (Jones *et al.* 1997). However, COX expression during the menstrual cycle is still not defined in monkey. The aim of this study was to examine immunohistochemically the expression of COX-1, COX-2, mPGES and cPGES proteins in monkey endometrium during the menstrual cycle.

Materials and Methods

Animals

Rhesus monkeys (*Macaca mulatta*) at the Fujian Province Non-Human Primate Research Center in China were caged individually in a controlled environment with a 12-h light–12-h dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (China). Animals were evaluated daily by visual examination of the perineum for menses, with the onset of menses defined as day 1 of the menstrual cycle. Adult female monkeys with regular menstrual cycles of approximately 28 days were chosen for this study. The uterine samples of the menstrual cycle were taken on days 6, 9 and 12 of the proliferative phase, and on days 16, 20 and 25 of the luteal phase. All of the monkeys were killed by an overdose injection of ketamine. At least three monkeys were

used in each group. The uteri were removed and treated as described below.

Immunohistochemistry

The dorsal portion of monkey uterus was immediately cut into small pieces, fixed in Bouin's solution for 24 h, dehydrated with graded ethanol and embedded in paraffin. Paraffin sections were cut, deparaffinized and hydrated gradually in PBS. For the sections used for COX-2 immunohistostaining, antigen retrieval was performed in 0.1 mM EDTA (pH 9.2) at 88 °C water bath for 10 min. Nonspecific binding was blocked with 10% horse serum in PBS at 37 °C for 1 h. The sections were incubated with goat anti-COX-1 immunoglobulin G (IgG) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-COX-2 IgG (1:300, Santa Cruz) at 24 °C for 90 min. Normal goat IgG was used to replace each primary antibody for a negative control. The sections were then incubated with biotinylated rabbit anti-goat antibody followed by an avidin–alkaline phosphatase complex and Vector Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA, USA). For the mPGES and cPGES immunostaining, the sections were incubated with rabbit anti-mPGES (1:200, Cayman Chemical, Ann Arbor, MI, USA) or rabbit anti-cPGES (1:400, Cayman Chemical) overnight at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit antibody followed by an avidin–alkaline phosphatase complex and Vector Red. Vector Red was visualized as a red color. Endogenous alkaline phosphatase activity was inhibited with levamisole (Sigma). The sections were counterstained with hematoxylin and mounted.

Additionally, normal rabbit IgG was used to replace the primary antibodies for a negative control. Moreover, rabbit anti-cPGES was preincubated with the blocking peptide for cPGES (Cat no. 360150, Cayman Chemical) for 30 min prior to incubating with the sections on day 25 of the menstrual cycle. Similarly, the rabbit anti-mPGES antibody was preincubated with mPGES blocking peptide (Cat No. 360140, Cayman Chemical) for 30 min prior to incubating with the sections on day 25 of the menstrual cycle.

The degree of staining was assessed subjectively by blinded examination of the slides by two investigators. A scoring system was designated as 0 = no stain, 1 = pale stain, 2 = moderate stain, 3 = strong stain and 4 = very intense stain. At least three monkeys at each stage were used under this analysis. The means $\pm$ S.E.M. of the staining intensity for each cellular compartment were shown graphically.

Results

COX-1 immunostaining

COX-1 immunostaining in monkey endometrium during the menstrual cycle is shown in Fig. 1 and scored in Fig. 2. COX-1 immunostaining was detected in the luminal

Figure 1 COX-1 immunostaining in monkey endometrium on days 6 (a), 9 (b), 12 (c), 16 (d), 20 (e) and 25 (f) of the menstrual cycle. Scale bars represent 30 μ m.

Figure 2 COX-1 immunostaining intensity in monkey endometrium during the menstrual cycle. Open bars, luminal epithelium; black bars, glands in functionalis; hatched bars, glands in basalis; gray bars, stroma (no bar, staining was undetectable in that part of the tissue). Each bar represents the mean $\pm$ S.E.M. of the immunostaining intensity.

epithelium throughout the menstrual cycle (Fig. 1). However, a high level of COX-1 staining was seen on days 12, 16 and 20 of the menstrual cycle, being at the highest level on day 16 (Fig. 1c–e). The level of COX-1 staining in the glandular epithelium was different between basalis and functionalis regions of the endometrium. No immunostaining was detected in the glandular epithelium of the basalis region, while a low level of COX-1 staining was detected in the glandular epithelium of the functionalis region except for day 9 of the menstrual cycle (Fig. 1b). There was no detectable COX-1 immunostaining in the stroma (Fig. 1). Additionally, there was no detectable COX-1 immunostaining when goat anti-COX-1 IgG was replaced by normal goat IgG (data not shown).

COX-2 immunostaining

COX-2 immunostaining in monkey endometrium during the menstrual cycle is shown in Fig. 3 and scored in Fig. 4.

There was no detectable COX-2 immunostaining in the endometrium on days 6 and 9 (Fig. 3a and b). A basal level of COX-2 immunostaining was seen in the luminal epithelium and glands in the functionalis region on day 12 (Fig. 3c). COX-2 immunostaining was strongly observed in the luminal epithelium and glandular epithelium of the functionalis region on day 16 (Fig. 3d). There was a low level of COX-2 immunostaining in the luminal epithelium and glandular epithelium of the functionalis region on day 20 (Fig. 3e). A basal level of COX-2 immunostaining was seen in the luminal epithelium and glandular epithelium of the functionalis region on day 25 (Fig. 3f). During the whole menstrual cycle, there was no detectable COX-2 immunostaining in the glandular epithelium of basalis regions or in the stroma (Fig. 3a–f). Additionally, there was no detectable COX-2 immunostaining when goat anti-COX-2 IgG was replaced by normal goat IgG (data not shown).

Figure 3 COX-2 immunostaining in monkey endometrium on days 6 (a), 9 (b), 12 (c), 16 (d), 20 (e) and 25 (f) of the menstrual cycle. Scale bars represent 30 μ m.

Figure 4 COX-2 immunostaining intensity in monkey endometrium during the menstrual cycle. Open bars, luminal epithelium; black bars, glands in functionalis; hatched bars, glands in basalis; gray bars, stroma (no bar, staining was undetectable in that part of the tissue). Each bar represents the mean $\pm$ S.E.M. of the immunostaining intensity.

Figure 5 cPGES immunostaining in monkey endometrium on days 6 (a), 9 (b), 12 (c), 16 (d), 20 (e) and 25 (f) of the menstrual cycle. (g) There was no detectable cPGES immunostaining in the endometrium on day 25 of the menstrual cycle when the rabbit anti-cPGES antibody was preincubated with cPGES blocking peptide. *Myometrium. Scale bars represent 35 μ m.

cPGES immunostaining

cPGES immunostaining in monkey endometrium during the menstrual cycle is shown in Fig. 5 and scored in Fig. 6. On day 6, a basal level of cPGES immunostaining was observed only in the luminal and glandular epithelium (Fig. 5a). On day 9, a low level of cPGES immunostaining was seen in the glandular epithelium of the basalis region and in the luminal epithelium, while only a basal level was observed in the glandular epithelium of the functionalis region and the entire stroma (Fig. 5b). On days 12, 16, 20 and 25, cPGES immunostaining was highly detected in the luminal epithelium, stroma and glandular epithelium located in both basalis and functionalis regions (Fig. 5c–f). However, when the rabbit anti-cPGES antibody was preincubated with cPGES blocking peptide, there was no detectable cPGES immunostaining in the endometrium on day 25 of the menstrual cycle (Fig. 5g (inset)).

mPGES immunostaining

mPGES immunostaining in monkey endometrium during the menstrual cycle is shown in Fig. 7 and scored in Fig. 8. There was a low level of mPGES immunostaining only in the luminal epithelium on day 6 (Fig. 7a). No immunostaining was seen in the luminal and glandular epithelium of functionalis region on days 9, 12 and 16 (Fig. 7b, d and e), while a basal level of mPGES immunostaining was seen in the glandular epithelium of the basalis region on days 9, 12 and 16 (Fig. 7c–e). No immunostaining was seen in the luminal epithelium on days 20 and 25 (Fig. 7f and h). On day 20, a low level of mPGES immunostaining was seen in the glandular epithelium of the functionalis region (Fig. 7f), while a high level in the glandular epithelium of the basalis region (Fig. 7g). On day 25, a high level of mPGES immunostaining was detected in the whole glandular epithelium (Fig. 7h and i). However, there was no detectable mPGES immunostaining in the glandular epithelium on day 25 of the menstrual cycle

when the rabbit anti-mPGES antibody was preincubated with mPGES blocking peptide (Fig. 7j (inset)).

Discussion

In this study, COX-1 immunostaining was mainly detected in the luminal epithelium and glandular epithelium near the lumen, the same as COX-1 localization in mouse, baboon, mink and human endometrium (Chakraborty *et al.* 1996, Song *et al.* 1998, Kim *et al.* 1999, Marions & Danielsson 1999). However, in ovine endometrium, COX-1 immunostaining was localized in both epithelial and stromal cells (Charpigny *et al.* 1997). COX-2 immunostaining was localized in the luminal and glandular epithelium in this study, like that in the western spotted skunk and sheep (Charpigny *et al.* 1997, Das *et al.* 1999). In human endometrium, COX-2 immunostaining was mainly seen in the human luminal epithelium and perivascular cells (Marions & Danielsson 1999). COX-2 was localized in the luminal epithelium and stroma in mouse, mink and baboon endometrium (Chakraborty *et al.* 1996, Kim *et al.* 1999). These findings suggest that the localization of COX-1 and COX-2 in the endometrium is slightly different among various species.

It has been shown that cPGES is predominantly linked with COX-1 to promote the immediate response, during which relatively high concentrations of arachidonic acid are released in a short period (Tanioka *et al.* 2000). In our study, cPGES was colocalized with COX-1 in the luminal epithelium and glandular epithelium, and both cPGES and COX-1 were highly expressed on days 16 and 20 of the menstrual cycle. In addition, culture studies have demonstrated that both human epithelial and stromal cells can synthesize PGE₂ *in vitro* (Smith & Kelly 1988). These data suggest that the coupling of cPGES and COX-1 in the luminal epithelium may be responsible for the synthesis of PGE₂ in monkey endometrium.

In this study, mPGES immunostaining was mainly detected in the glandular epithelium on days 20 and 25 of

Figure 6 cPGES immunostaining intensity in monkey endometrium during the menstrual cycle. Open bars, luminal epithelium; black bars, glands in functionalis; hatched bars, glands in basalis; gray bars, stroma (no bar, staining was undetectable in that part of the tissue). Each bar represents the mean $\pm$ S.E.M. of the immunostaining intensity.

Figure 7 mPGES immunostaining in monkey endometrium on days 6 (a), 9 (b and c), 12 (d), 16 (e), 20 (f and g) and 25 (h and i) of the menstrual cycle. (j) There was no detectable mPGES immunostaining in the glandular epithelium on day 25 of the menstrual cycle when the rabbit anti-mPGES antibody was preincubated with mPGES blocking peptide. *Myometrium. Scale bars represent 25 μ m.

the menstrual cycle. In human endometrium, mPGES immunoreactivity in the functionalis layer was expressed in glandular epithelial, stromal, and endothelial cells (Milne *et al.* 2001). mPGES is preferentially coupled with

the inducible COX-2 to promote delayed PGE₂ generation and, if COX-2 already exists in cells, it also regulates immediate PGE₂ generation (Jakobsson *et al.* 1999, Murakami *et al.* 2000). In the present study, COX-2

Figure 8 mPGES immunostaining intensity in monkey endometrium during the menstrual cycle. Open bars, luminal epithelium; black bars, glands in functionalis; hatched bars, glands in basalis; gray bars, stroma (no bar, staining was undetectable in that part of the tissue). Each bar represents the mean $\pm$ S.E.M. of the immunostaining intensity.

immunostaining was strongly detected in the luminal epithelium and glandular epithelium on day 16, and weakly on day 20. However, mPGES immunostaining was strongly seen only in the glandular epithelium on days 20 and 25. The expression peak of COX-2 was not matched with that of mPGES. COX-2 was colocalized with mPGES in the glandular epithelium only on day 20 of the menstrual cycle, which was within the potential implantation window. Nevertheless, it is important to see whether COX-2 and mPGES are highly coexpressed in the endometrium of rhesus monkey during the peri-implantation period. During early pregnancy in mice, both COX-2 and mPGES were highly expressed at implantation sites on day 5 of pregnancy (Chakraborty *et al.* 1996, Ni *et al.* 2002). Furthermore, COX-2-deficient mice had multiple failures in female reproductive processes, including ovulation, fertilization, implantation and decidualization (Lim *et al.* 1997). Conversely, Cheng and Stewart (2003) reported that COX-2-deficient mice did not show any abnormalities in embryo implantation except for the reduction in the numbers of ovulated and fertilized eggs, and a delay in decidual growth. This difference may be caused by the different genetic backgrounds of the COX-2-deficient mice used by the two groups. The role of COX-2 during embryo implantation also needs to be further investigated.

The exact role of PGE₂ in the monkey endometrium is not fully elucidated. It seems that COX-1 and cPGES are mainly responsible for the synthesis of PGE₂ in the monkey endometrium during the menstrual cycle. In bovine endometrium, COX-1 mRNA and protein are not expressed on any day of the estrous cycle, whereas COX-2 mRNA, and protein and mPGES mRNA are differentially expressed and regulated in bovine endometrium during the estrous cycle. The COX-2 and mPGES pathway is responsible for the endometrial production of PGE₂ in the bovine endometrium during the estrous cycle (Arosh *et al.* 2002). In human colon epithelial cells, overexpression of COX-2 and enhanced synthesis of PGE₂ have been shown to promote the proliferation and survival of cells through

inhibition of apoptosis. PGE₂ has been shown to upregulate expression of antiapoptotic genes such as bcl-2 (Sheng *et al.* 1998). It is plausible that, in the human endometrium, PGE₂ may be activating similar mechanisms that promote glandular epithelial cell proliferation and/or survival (Milne *et al.* 2001). PGE₂ function in glandular epithelial cells may also be associated with the regulation of uterine angiogenesis. In the human endometrium, vascular endothelial growth factor expression is localized to the glandular epithelial cells throughout the menstrual cycle (Torry *et al.* 1996). Moreover, PGE₂ has been previously shown to upregulate the expression of vascular endothelial growth factor in a number of different cell types (Harada *et al.* 1994, Cheng *et al.* 1998). Nevertheless, the availability of arachidonic acid may be a rate-limiting step in synthesizing PGE₂. Cytosolic phospholipase A₂ α (cPLA₂ α) is a major provider of arachidonic acid in the COX system for PG biosynthesis. cPLA₂ α deficiency results in aberrant uterine spacing of embryos and deferred implantation (Song *et al.* 2002). It remains to determine whether arachidonic acid is provided for PGE₂ synthesis through the COX-1/cPGES and/or COX-2/mPGES pathway.

In conclusion, our data suggest that the coupling of cPGES and COX-1 in the luminal epithelium may be responsible for the synthesis of PGE₂ in monkey endometrium, and the coupling of mPGES and COX-2 in the glandular epithelium may be important in preparing the receptive endometrium.

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