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The effect of prostaglandin E_2 receptor (PTGER2) activation on growth factor expression and cell proliferation in bovine endometrial explants

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ABSTRACT

The domestic animal endometrium undergoes regular periods of regeneration and degeneration during cycles of oestrus and dioestrus. If blastocyst implantation occurs in the uterus, the endometrium will prepare for pregnancy by changing its pattern of development to build a connection with the embryo to nourish it. Prostaglandin E_2 (PGE_2) secretion synchronized with endometrial growth in these processes and could be involved in endometrial growth. One of the PGE_2 receptors (PTGER2) is present in endometrium and its increased expression accompanies with endometrial growth in above processes. However, the association between PTGER2 and endometrial growth remains unclear. Endometrial growth factors and cell proliferation is the foundation for endometrial growth. Therefore, in this study, the response of growth factors and cell proliferation essential for endometrial growth to PTGER2 activation were investigated in bovine endometrium. The results indicated that mRNA and protein expression of connective tissue growth factor (CTGF), fibroblast growth factor-2 (FGF-2), interleukin-8 (IL-8), transforming growth factor-β1 (TGF-β1), matrix metalloproteinase-2 (MMP-2), and vascular endothelial growth factor A (VEGFA) were up-regulated after PTGER2 activation by corresponding agonist butaprost ($P < 0.05$), and proliferation of endometrial epithelia and fibroblasts were induced in response to increased levels of proliferating cell nuclear antigen (PCNA), cytokeratin-18 (CK-18) and fibroblast-specific protein 1 (FSP-1) expression in bovine endometrial explants in vitro ($P < 0.05$).

1. Introduction

The domestic animal endometrium undergoes histological and physiological changes that is necessary for blastocyst implantation, with a remarkable capacity for self-repair during oestrous cycles. Endometrium thickens from pro-oestrus, with the endometrium growing gradually through mid-oestrus and late-oestrus periods. Degeneration of the endometrium occurs during dioestrus. After blastocyst implantation occurs in the uterus, the endometrium will prepare for pregnancy by changing its pattern of development to build a connection with the embryo to nourish it. These changes are directly and indirectly caused by steroid hormones such as estrogen (E_2) and progesterone (P_4), as well as cytokines synthesized and released by epithelial, stromal, and vascular cells of the endometrium [1]. However, the specific mechanism of endometrial growth during oestrus period and peri-implantation remains unclear.

Prostaglandin E_2 (PGE_2) production begins when phospholipases liberate arachidonic acid (AA) stored in membrane phospholipids. AA is then converted into prostaglandin endoperoxide H_2 (PGH_2) by cyclooxygenases (COXs). Two COX isoforms, types 1 and 2 catalyse the double oxygenation and reduction of AA. PGH_2 is then transformed into PGE_2 via PGE synthase (PGEs). PGE_2 mediates its effect via binding four receptor subtypes, PTGER1, PTGER2, PTGER3, and PTGER4 [2].

Capacity of PGE_2 regulating mitosis, anti-apoptosis, angiogenesis, cell proliferation and tissue remodelling has been demonstrated [3–5]. Maybin et al. indicated that PGE_2 could regulate growth factor expression and cell proliferation in human endometrium during proliferative phase, but which PTGERs involved in this process was unknown [6,7]. PTGER2, localized in epithelium, stromal cells, and glandular epithelial cells of bovine endometrium, exhibited increased protein expression during oestrus period when endometrial growth happens, compared with that in dioestrus [8]. What's more, PTGER2 plays a positive role in endometrial growth during endometriosis and endometrial cancer, such as, PTGER2 involved in endometrial

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adenocarcinoma development by regulating growth factors expression [9], PTGER2 inhibition suppresses adhesion, invasion, proliferation of endometriotic epithelial cells and stromal cells, induces apoptosis of endometriotic cells, and suppresses the growth and survival of endometriosis lesions [10–14]. Shi et al. suggested that PTGER2 expression at implantation site plays an important role during blastocyst implantation in rats [15]. Based on these results, we hypothesized that PTGER2 might be involved in endometrial growth through regulating growth factors expression and cell proliferation during oestrous cycle and peri-implantation in ruminants.

The aim of the present study was to investigate the response of endometrial growth factors and endometrial epithelia and fibroblasts to PTGER2 activation in bovine endometrium. In the experiment, mRNA and protein expression of endometrial growth factors including CTGF, FGF-2, IL-8, MMP-2, TGF-β1, and VEGFA as well as proliferation of epithelia and fibroblasts were measured after PTGER2 activated by PTGER2 selective agonist butaprost ($10^{-9}-10^{-5}$ M) in bovine endometrial explants in vitro.

2. Materials and methods

2.1. Reagents, chemicals and antibodies

The following reagents were purchased from the indicated manufacturers: Fetal bovine serum (ExCell Biology Inc., China); Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco, USA); butaprost (CAYMAN, USA); Primary antibodies, including rabbit anti-CTGF antibody, mouse anti-proliferating cell nuclear antigen (PCNA) antibody, mouse anti-cytokeratin-18 (CK-18) antibody, mouse anti-IL-8 antibody, mouse anti-MMP-2 antibody, rabbit anti-fibroblast-specific protein-1 (FSP-1) antibody, rabbit anti-caspase-3 antibody, and rabbit anti-TGF-β1 antibody (Abcam, UK); rabbit anti-VEGFA antibody (SANTA CRUZ, USA); rabbit anti-FGF-2 antibody (NovusBio, USA); Secondary antibodies, including goat anti-rabbit IgG horseradish peroxidase (HRP)-linked and goat anti-mouse IgG HRP-linked (Cell Signaling Technology, USA); Goat anti-mouse IgG H & L antibody (Alexa Fluor® 488) and donkey anti-Rabbit IgG H & L antibody (Alexa Fluor® 647) (Abcam, UK); Rabbit IgG-isotype (Abcam, UK); and mouse IgG-isotype (R & D Systems, USA). The primers were synthesized by Invitrogen, China.

2.2. Collection and cultivation of endometrial tissue in vitro

The animal studies were conducted in accordance with the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Inner Mongolia Agricultural University. Fresh and healthy Holstein bovine bilateral uterine horns close to the ovary in the pro-oestrus stage (hormone levels in pro-oestrus endometrium is basal) were obtained from a local abattoir (n = 8 cows) according to ovarian morphology [16–18]. Endometrial tissue was dissected and processed as described previously [19]. In briefly, the tissues were washed thrice with phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin and streptomycin and 2.5 μg/ml amphotericin B and were stored at 4 °C for 1 h. Then, each uterine horn was excised under aseptic conditions and opened longitudinally, and endometrial tissue containing epithelia and stroma was obtained from the endometrial region using curved scissors and ophthalmic tweezers. Endometrial tissues were subdivided into pieces approximately 2 mm in diameter and 1 mm in thickness; each of the 8 explants was then randomly placed in one well of a 6-well culture plate with 5 ml of medium (DMEM/F-12 with 20% fetal bovine serum, 100 IU/ml penicillin and streptomycin and 2.5 μg/ml amphotericin B). Endometrial explants were incubated in 95% air plus 5% CO_2 at 37 °C. At 24 h intervals, the medium was replaced until treatment at 48 h.

2.3. Experimental treatment

The bovine endometrial fragments were cultured for 48 h and then separated into the following groups: concentration-effect experiment, including a control group (butaprost diluent, ethanol) and butaprost treatment group ($10^{-9}-10^{-5}$ M); time-effect experiment, including a control group (butaprost diluent, ethanol) and butaprost treatment group (10^{-7} M) for indicate time. Butaprost concentration was determined by Ki value of PGE_2 for PTGER2 is about 10^{-8} M and bovine endometiral PGE_2 physiology concentration is about $10^{-9}-10^{-8}$ M [2,20], and Ki value of butaprost for PTGER2 is about 10^{-7} M [21]. The collected explants were washed with PBS, snap-frozen in liquid nitrogen, and stored at − 80 °C until mRNA and protein extraction at the indicated times. The explants that had been treated with butaprost (10^{-7} M) and butaprost diluent (ethanol) for 24 h were washed with PBS, soaked in optimal cutting temperature (O.C.T.) compound and stored at − 80 °C prior to Immunofluorescence analysis.

2.4. Real-time RT-PCR analysis

Total mRNA extraction, reverse transcription and real-time RT-PCR were conducted according to the manufacturers' instructions (for mRNA extraction, AxyPrep™ Multisource Total RNA Miniprep Kit, Axygen Scientific Inc.; for reverse transcription, PrimeScript™ RT Master Mix, Takara Bio Inc.; for RT-PCR, FastStart Universal SYBR Green Master, Roche Applied Science). Reverse transcribed cDNA obtained from total RNA of treatment and control groups was amplified as follows: denaturing at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s on a ABI ViiA 7 RT- PCR system (Applied Biosystems). The primers used for real-time RT-PCR are given in Table 1. β-actin was used as an internal control. Results are presented as $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct - \Delta Ct_{control}$ and $\Delta Ct = Ct_{target} - Ct_{\beta\text{-actin}}$).

2.5. Western blot analysis

Total protein extraction, concentration measurements and protein denaturation were conducted according to the manufacturers' instructions (for protein extraction, T-PER™ Tissue Protein Extraction Reagent, Thermo Fisher Scientific; for protein concentration measurement, Pierce™ BCA protein Assay Kit, Thermo Fisher Scientific; for protein denaturation, SDS-PAGE Sample Loading Buffer, Beyotime). Denatured proteins were stored at −80 °C. Then, 12% SDS-PAGE gel electrophoresis was performed at 80 V with a protein sample loading quantity of 20 μg. The semi-dry transfer conditions were 40 V for 30 min. The membrane was blocked for 1 h at room temperature (RT) and incubated in primary antibody at 4 °C for 14 h. The primary antibody dilutions were as follows: CTGF 1:1000, FGF-2 1:2000, IL-8 1:1000, MMP-2

Table 1
Primers used in this study.

Gene symbol	Accession No.	Primer sequence
β-actin	NM_173979.3	Forward: 5′-CCAAGGCCAACCGTGAGAAGAT-3′ Reverse: 5′-CCACGTTCCGTGAGGATCTTCA-3′
CTGF	NM_174030.2	Forward: 5′-AGCTGACCTGGAGGAGAACA-3′ Reverse: 5′-GTCTGTGCACACTCCGCAGA-3′
FGF-2	NM_174056.4	Forward: 5′-TGTTATGCCGAGTTGCTCAG-3′ Reverse: 5′-TGCCTACAATGAGGGAGACC-3′
IL-8	NM_173925.2	Forward: 5′-CCTCTTGTTCAATATGACTTCCA-3′ Reverse: 5′-GGCCCACTCTCAATAACTCTC-3′
MMP-2	NM_174745.2	Forward: 5′-GGCATCTCTCAGATCCGTGG-3′ Reverse: 5′-TGTGGGTCTTCGTACACAGC-3′
TGF-β1	NM_001166068.1	Forward: 5′-CTGTGGCTGCTAATGCTGAC-3′ Reverse: 5′-GTGACCTCCTTGGCGTAGTA-3′
VEGFA	NM_001316955.1	Forward: 5′-GACCCTGGTGGACATCTTCC-3′ Reverse 5′-CACACAGGGCACACACTCC-3′