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■ Brief Communication ■

Endothelial Cells Isolated from the Bovine Corpus Luteum Synthesize Prostaglandin $F_{2\alpha}$ Receptor

Sun-Yeong Gwon[1], Ki-Jong Rhee[1] and Seunghyung Lee[2,†]

[1]Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University at Wonju, Wonju, Gangwon-do 220-710, Korea

[2]Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA 19140, USA

The corpus luteum is a transient endocrine gland essential for regulation of the ovarian cycle as well as for establishing and maintaining pregnancy. Prostaglandin $F_{2\alpha}$ (PGF) initiates functional and structural regression of the corpus luteum and therefore is an important regulator of the estrous cycle. It is a matter of debate whether the endothelial cells of the bovine corpus luteum express PGFR, the cognate receptor for PGF. Therefore, the aim of this study was to assess the expression of PGFR in bovine endothelial cells. Endothelial cells were isolated from the bovine corpus luteum of the mid-luteal stage using magnetic beads and cultured *in vitro*. We demonstrate that this isolation procedure generates a pure culture of endothelial cells as confirmed by synthesis of Factor VIII and lack of expression of 3β-hydroxysteroid dehydrogenase. By RT-PCR, Western blot and immunofluorescence analyses, we further show that the cultured endothelial cells produced PGFR. This model system can be utilized to provide an experimental system to investigate the role of PGF on endothelial cells during the reproductive cycle.

Key words: Prostaglandin $F_{2\alpha}$ (PGF), PGF receptor (PGFR), Endothelial cell, Corpus luteum

The corpus luteum is a transient endocrine gland essential for regulation of the ovarian cycle as well as for establishing and maintaining pregnancy. During the bovine estrous cycle, the corpus luteum is present for 17-18 days (Niswender et al., 2000). It is universally accepted that pulsatile release of prostaglandin $F_{2\alpha}$ (PGF) from the endometrium initiates functional and structural regression of the corpus luteum (Silvia et al., 1991). The corpus luteum consists of several cell types with vascular endothelial cells accounting for up to 50% of the total cells whereas luteal steroidogenic cells account for 30% and other cells account for 20% of cells (Lei et al., 1991). In mammalian reproductive tissues, PGF is predominantly secreted from the uterus and ovary and is responsible for the regression of corpus luteum at the end of the estrous cycle (Horton and Poyser, 1976; Hansel and Dowd, 1986). Therefore, PGF is regarded as a physiological luteolysin.

PGF binds to PGF receptor (PGFR) expressed by bovine luteal cells throughout the estrous cycle (Rao et al., 1979; Anderson et al., 2001). Contradictory reports exist regarding the presence of PGFR in luteal endothelial cells. Wiltbank et al. reported that PGFR is expressed in luteal tissue by both the luteal steroidogenic cells and by the large blood vessels in the peripheral region of the mature corpus luteum (Wiltbank et al., 1995). Two studies have suggested that luteal steroidogenic cells from bovine corpus luteum express PGFR (Wiltbank et al., 1995; Anderson et al., 2001). In regards to luteal endo-

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†Corresponding author: Seunghyung Lee. Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center Temple University School of Medicine, Philadelphia, PA 19140, USA.
Tel: +1-215-707-8015, Fax: +1-215-707-2783
e-mail: S.Lee@temple.edu

thelial cells, several studies suggest that freshly isolated luteal endothelial cells from bovine corpus luteum do express PGFR (Mamluk et al., 1998; Meidan et al., 2005) whereas Cavicchio et al. reported an absence of PGFR expression in purified luteal endothelial cells along with lack of functional responses to PGF (Cavicchio et al., 2002). Therefore, it is still unclear whether PGFR protein is synthesized in cultured bovine luteal endothelial cells. In the present study, we examined *PGFR* mRNA expression and PGFR protein synthesis in isolated endothelial cells from bovine corpus luteum.

To examine if luteal endothelial cells synthesized *PFGR*, we initially determined the localization of PGFR expression in corpus luteum by immunohistochemistry. The bovine corpus luteus was collected within 10-20 min after exsanguinations and transported to the laboratory on ice. The corpus luteum at mid-luteal stage was determined by macroscopic observation of the ovary and uterus (Miyamoto et al., 2000). Tissues were fixed with 10% formalin and then embedded in paraffin. Tissue sections (4 μm) were treated with antigen retrieval buffer (0.01 M citrate acid, pH 6.0), blocked with 10% normal goat serum and subsequently stained with anti-PGFR antibody (Abcam, Cambridge, MA, USA). Thereafter, the tissues were stained sequentially with biotinylated anti-rabbit IgG antibody, avidin-biotin-peroxidase complex, DAB and counterstained with hematoxylin (all from Vector Laboratories, Burlingame, CA, USA). We found that PGFR is expressed by both luteal steroidogenic cells and endothelial cells in the bovine corpus luteum at mid-luteal stage (Fig. 1A).

To determine if isolated endothelial cells retained their ability to produce PGFR, the cells were isolated using *Bandeiraea simplicifolia* lectin-conjugated magnetic beads. Briefly, corpus luteum were enzymatically dissociated using collagenase and DNase Ⅰ, then cells were filtered through metal meshes. Thereafter, cells were mixed with magnetic beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) coated with *Bandeiraea simplicifolia* (Sigma, Saint Louis, MO, USA) which specifically binds glycoproteins expressed by bovine endothelial cells (Labarca and Paigen, 1980; Klipper et al., 2004). Bound cells were eluted with fucose solution and were seeded in collagen coated tissue culture plates containing DMEM/F-12 (Cellgro, Manassas, VA, USA) containing 20% fetal bovine serum, 5% microvascular growth supplement (Gibco, Grand Island, NY, USA) and antibiotics. The endothelial cells showed a homogenous morphology at the beginning of culture (Fig. 1B) and reached confluence within 3-5 days. At this time, the monolayer ex-

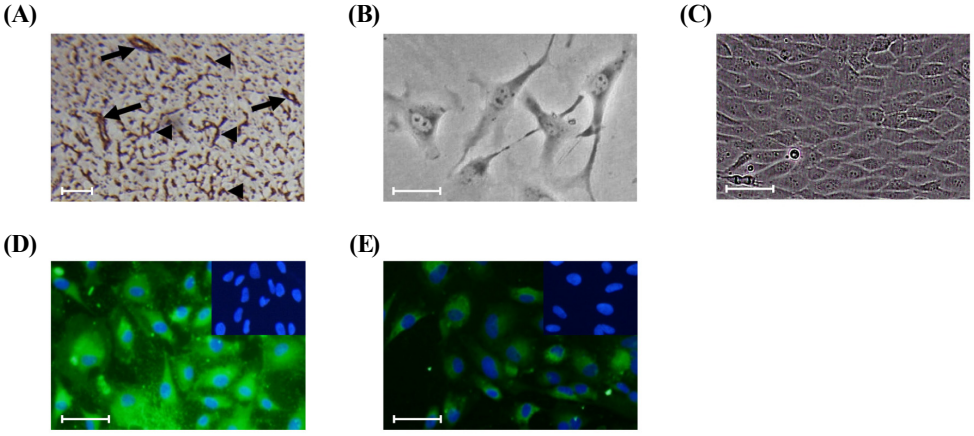

Fig. 1. Histological images of luteal endothelial cells. (A) Immunohistochemical image of PGFR expressing cells of the corpus luteum. Corpus luteum at day 8-12 of the estrous cycle was stained with anti-PGFR antibodies (brown). The localization of luteal steroidogenic cells (arrowheads) and endothelial cells (arrows) are indicated. Bar, 40 μm. (B) Freshly isolated endothelial cells prior to culture examined with phase contrast microscopy. Bar, 40 μm. (C) Endothelial cells cultured until passage 5 showing cobblestone morphology examined with phase contrast microscopy. Bar, 20 μm (D) Immunofluorescence image of Factor VIII in cultured endothelial cells (green) and DAPI (blue). Inset, isotype matched control. Bar, 40 μm. (E) Immunofluorescence image of PGFR in cultured endothelial cells (green) and DAPI (blue). Inset, isotype matched control. Bar, 40 μm. All data are representative of three separate experiments (n=3).

hibited a cobblestone-like morphology typical of endothelial cell cultures (Fig. 1C). This morphology is comparable to cultured endothelial cells isolated from the bovine aorta or vena cava caudalis (Fenyves et al., 1993; Spanel-Borowski et al., 1994). Upon further examination, the cultured endothelial cells in the current study corresponded to type 1 endothelial cells according to the classification scheme reported by Spanel-Borowski and Bosch (Spanel-Borowski and van der Bosch, 1990).

To confirm the purity of the cultured endothelial cells, we examined the cultured cells using immunofluorescence staining of the classical endothelial cell marker, Factor VIII (also known von Willebrand factor). Cultured cells were fixed with 3.7% paraformaldehyde and then stained with anti-human Factor VIII antibody (Sigma) and Cy3 labeled donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA). Slides were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories) and observed using a Nikon-Eclipse E1000 fluorescent microscope. All cells were stained positively by anti-Factor VIII antibodies suggesting that pure cultures of endothelial cells were isolated (Fig. 1D).

To identify if the cultured endothelial cells synthesized *PFGR*, immunofluorescence microscopy was performed on the cultured cells using anti-PGFR antibody followed by Cy3-labeled donkey anti-rabbit antibody and DAPI. We found that all cells were positive for *PFGR* suggesting that the cultured endothelial cells synthesized *PFGR* (Fig. 1E). Cells stained with isotype matched control antibodies were negative. To verify that the cultured endothelial cells expressed and synthesized *PFGR*, we used RT-PCR and Western blot analysis, respectively. Total RNA was extracted using TRIzol® and cDNA was synthesized using murine Moloney leukemia virus reverse transcriptase (MMLV-RT; Invitrogen). cDNA was PCR amplified using specific primers for PGFR (forward-TTA GAA GTC AGC AGC ACA G & reverse-ACT ATC TGG GTG AGG GCT GAT T) and *β-actin* (forward-CGG CAT TCA CGA AAC TAC C & reverse- ATC AAG TCC TCG GCC ACA C). We found that cultured endothelial cells expressed *PFGR* mRNA (Fig. 2A). In addition, PGFR synthesis was confirmed by Western blot analysis. Tissues were lysed, subjected to SDS-PAGE under reducing conditions and sequentially stained with anti-PGFR antibody. We found that cultured endothelial cells synthesized *PFGR* by Western blot analysis (Fig. 2B).

Finally, to confirm the purity of the endothelial cells at the molecular level we employed RT-PCR analysis. Luteal steroidogenic cells express 3β-hydroxysteroid dehydrogenase whereas endothelial cells in corpus luteum do not (Maybin and Duncan, 2004). cDNA from cultured endothelial cells were amplified with specific primers for *3β-HSD* (5` TCC ACA CCA GCA CCA TAG AA & reverse-CTC CTT GGT TTT CTG CTT GG). We found that whereas the total luteal cells were positive for *3β-HSD* expression, cultured endothelial cells were negative for *3β-HSD* expression (Fig. 2C). Taken together, these results strong argue that the endothelial cell isolation procedure produced a pure culture of endothelial cells which express the PGFR mRNA and protein.

The present study provides further evidence that PGFR is present on microvasculature endothelial cells in bovine luteal endothelial cells. These results are in agreement with studies that suggested that PGFR is expressed by both luteal steroidogenic cells and endothelial cells of bo-

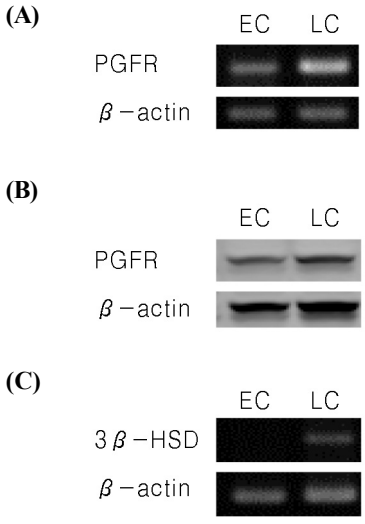

Fig. 2. Analysis of PGFR and *3β-HSD* expression and synthesis. Expression of PGFR and *3β-HSD* was examined in cultured endothelial cells (EC) and total luteal cells (LC). RT-PCR analysis (A, C) and Western blot analysis (B). All data are representative of three separate experiments (n=3).

vine corpus luteum collected at the mid-luteal stage (Meidan et al., 2005; Shirasuna et al., 2012). The data from the current study strongly suggest that PGF can act directly on luteal endothelial cells both *in vivo* and *in vitro* as suggested by other investigators (Girsh et al., 1996; Lee et al., 2009). PGFR expression has been found in luteal endothelial cells not only in cows but also in ewes (Ristimaki et al., 1997) and pigs (Zannoni et al., 2007). However, studies by Cavicchio et al. and Liptak et al. reported an absence of *PGFR* mRNA in bovine luteal microvascular endothelial cells (Cavicchio et al., 2002; Liptak et al., 2005). The discrepancy from our results and the aforementioned studies may lie in the stage of corpus luteum used, different techniques utilized and the purity of the luteal endothelial cells (Spanel-Borowski, 2011).

In conclusion, we demonstrate that cultured luteal endothelial cells express PGFR. The isolated technique used in the current study allows for isolated and expansion of a pure culture of luteal endothelial cells to allow investigations of on bovine luteal endothelial cells *in vitro*. This culture model can further our understanding of the factors and mechanisms responsible for regulation of PGF and PGFR gene expression during luteal development and regression.

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