Stimulation of tube formation mediated through the prostaglandin EP2 receptor in rat luteal endothelial cells

Toshihiro Sakurai, Kenta Suzuki, Mikihiro Yoshie, Keisuke Hashimoto, Eiichi Tachikawa and Kazuhiro Tamura

Department of Endocrine Pharmacology, Tokyo University of Pharmacy and Life Sciences, Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan
(Correspondence should be addressed to K Tamura; Email: hiro@toyaku.ac.jp)
T Sakurai is now at Laboratory of Animal Breeding, Faculty of Agriculture, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Abstract

To explore the role of prostaglandin E2 (PGE2) in angiogenesis in the developing corpus luteum, luteal microvascular endothelial-like cells (luteal ECs) were prepared from highly luteinizing ovaries of rats using the percoll density gradient method. The cells abundantly expressed the mRNAs of the endothelial markers CD31 (PECAM-1) and responded to the vascular endothelial growth factor (VEGF) to form in vitro tube structures on Matrigel. Cyclooxygenase (COX) inhibitors significantly suppressed tube formation in luteal ECs, whereas PGE2 counteracted the COX inhibitor-induced blockage. PGE2-induced tube formation was blocked by a cyclic AMP-dependent protein kinase A (PKA) inhibitor, H89. The antagonist against the PGE receptor type 2 (EP2 receptor), AH6809, completely inhibited PGE2-induced tube formation and partly suppressed the VEGF-induced tube formation but did not attenuate PGE2-induced phosphorylation of both AKT kinase and extracellular signal-regulated kinase 1/2. VEGF significantly enhanced the expression of COX-2 mRNAs detected by real-time RT-PCR and PGE2 secretion into the media measured by ELISA in luteal ECs. In turn, PGE2 stimulated VEGF expression. In vitro co-culture of luteal ECs with steroidogenic luteal cells (SLCs) promoted tube formation. Pre-treatment of SLCs with VEGF further enhanced tube formation of ECs, and this effect was blocked by the COX-2 inhibitor. This stimulatory effect was inhibited by treatment with AH6809. These data indicate that PGE2 exerts a direct stimulatory effect on tube formation mainly via the EP2 receptor/PKA signaling in luteal ECs. Our results suggest the possibility that the endogenous PGE2 that is produced from luteinizing follicular cells as well as ECs may stimulate luteal angiogenesis.

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Introduction

Granulosa cells and theca cells in the Graafian follicles rapidly transform into luteal cells after ovulation in humans and rodents. The corpus luteum (CL) secretes a large amount of progesterone (P4) for the establishment of pregnancy. The acquirement of luteal function requires the formation of new capillary vessels that invade from the theca cells of the follicular wall during initiation of the development of the CL (Findlay 1986, Reynolds & Redmer 1998). The mature CL is a highly vascularized transient tissue, and the number of microvascular endothelial cells (ECs) in this tissue exceeds 50% of the total number of cells (Reynolds et al. 2002). The prominent vascularization of the CL may provide luteal cells with abundant cholesterol, which is a precursor of P4 synthesis and promotes the delivery of P4 into circulation. Various angiogenic factors that are derived from granulosa cells of the ovulated follicles probably promote the expression of the follicular capillary bed in the theca interna. Impaired normal vascular formation may cause insufficiency of the CL in the luteal phase or in the early stage of pregnancy.

The vascular endothelial growth factor (VEGF) plays a critical role in the regulation of the development and function of the CL (Ferrara et al. 1998, Kaczmarek et al. 2005). VEGF is a major regulator of endothelial proliferation and migration (Ferrara & Davis-Smyth 1997) and is closely related to the growth of capillary vessels in the CL (Kaczmarek et al. 2005). The expression of ovarian VEGF is markedly increased during the formation of the CL and is mostly localized to steroidogenic luteal cells (SLCs) in rats (Shweiki et al. 1993, Kashida et al. 2001). Moreover, a VEGF receptor blocker interferes with luteal vascular proliferation (Pauli et al. 2005).

Prostaglandins (PGs) are critical regulators of ovarian function, particularly with regard to ovulation and luteal regression (Olofsson & Leung 1996, Arosh et al. 2004). Their role in luteal development, however, is not well characterized. Our previous study showed that the activity of cyclooxygenase 2 (COX-2) is associated with the formation of a
functional CL via the stimulation of angiogenesis in vivo (Sakurai et al. 2003), and that VEGF stimulates prostaglandin E2 (PGE2) production by stimulating COX-2 expression in cultured rat luteal cells (Sakurai et al. 2004). In the ovary, the concentration of PGE2 is increased in the follicular fluid immediately before and after ovulation in Graafian follicles (Brown & Poyer 1984, Arosh et al. 2004). PGE2 can modulate vascular permeability and angiogenesis (Nie & Honn 2004). PGE2 upregulates VEGF mRNA at the transcriptional level in rat osteoblasts and in bone tissues (Harada et al. 1994), which suggests the involvement of VEGF in the PG-mediated stimulation of bone formation. Furthermore, PGE2 stimulates the production and signaling of VEGF in human vascular ECs (HUVESCs; Tamura et al. 2006). Four G protein-coupled receptors have been identified for PGE2: EP1, EP2, EP3, and EP4 (Bos et al. 2004). These subtypes differ in their signal transduction pathways: EP1 is coupled to Ca2+ mobilization, EP2 and EP4 coupling to Gs protein are mainly associated with cyclic AMP (cAMP) formation, and EP3 is coupled to the inhibition of adenylate cyclase. Several reports support the critical role of PGs in angiogenesis via the EP receptors (Chang et al. 2004, Sung et al. 2005). However, the role of PGE2 in the functions of luteal ECs remains unknown. To address the role of PGE2 in physiological angiogenesis in the CL, we show in this study the effect of a COX inhibitor and PGE2 on the functions of ECs derived from rat luteinizing ovarian cells.

**Materials and Methods**

**Reagents**

The MCDB131 medium, EC growth supplement (ECGS), dibutryl-cyclic AMP (db-cAMP), and EP prostaglandin receptor antagonists (EP1 blocker: SC19220; EP2 blocker: AH6809; EP4 blocker: AH23848) and EP3 agonist (sulprostone) were purchased from Sigma–Aldrich. Recombinant human VEGF165 and PGE2 were obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and Cayman Chemical (Ann Arbor, MI, USA) respectively. The selective inhibitors of COX-1 (SC-560) and COX-2 (NS-398) were also obtained from Cayman Chemical. The VEGF receptor-2 inhibitor (SU5614) and H89 were purchased from Calbiochem (San Diego, CA, USA) and D. Western Therapeutics Institute, Inc. (Nagoya, Japan) respectively. The growth factor-reduced MATRIGEL matrix (Matrigel; BD Biosciences, Bedford, MA, USA) was gelatinated in 24-well plates at 37 °C in a CO2 incubator for 30 min before in vitro angiogenesis assay. Total RNA and poly(A)+ RNA were extracted using Isogen (Nippon Gene Co., Ltd, Tokyo, Japan) and the QuickPrep micro mRNA purification kit (GE Healthcare, Buckinghamshire, UK) respectively.

Isolation, culture, and characterization of luteal ECs

All rat experimental protocols used in this study were reviewed and approved by the Institutional Animal Care Committees at the Tokyo University of Pharmacy and Life Sciences and were in compliance with the institutional guidelines for experimental animal care (#0827). Immature Wistar-Imamichi female rats were injected s.c. with 50 IU equine chorionic gonadotropin (eCG; ASKA Pharmaceutical Co. Ltd, Tokyo, Japan) at 0900 h on day 24 and were then injected i.p. 54 h later (i.e. 1500 h on day 26) with 25 IU human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co. Ltd) to induce superovulation and produce highly luteinized ovaries. After 2 days, ovaries were collected and digested for 1 h in Ca2+-Mg2+-free PBS (CMF-PBS) containing collagenase (Type I; 1 mg/ml, Sigma–Aldrich) and DNase (0-4 mg/ml) with shaking at 37 °C. Dispersed ovarian cells were then passed through a 70 μm nylon cell strainer (BD Biosciences), and the passed cells were washed with CMF-PBS. We used a modified version of the method that has successfully been used to isolate microvascular ECs from the mouse skin (Cha et al. 2005) to collect the luteal EC preparation. Briefly, whole luteinized ovarian cells were cultured for 2 days in MCDB131 medium (i.e. a basal medium for EC culture) containing 10% fetal bovine serum (FBS, JRH Biosciences, ACSL, Lenexa, MD, USA), ECGS (50 μg/ml), and antibiotics (50 μg/ml penicillin and streptomycin, 100 μg/ml gentamycin and neomycin, and 0.5 μg/ml amphotericin-B) in a 95% air/5% CO2 humidified atmosphere. On the second day of cell culture (70–80% confluence), cells were harvested using the standard method of trypsinization and centrifuged at 200 g for 5 min. The cell pellet was resuspended in 200 μl of 0-9% NaCl. Concurrently, a sterile gradient of Percoll was prepared by centrifuging a 35% percoll solution (Amersham Biosciences) at 30 000 g for 15 min at 4 °C. The dispersed cells (~4×107 cells) were layered onto the gradient percoll solution. A tube with a percoll gradient layered with a mixture of color density marker beads (Amersham Biosciences) was used as a density reference. The tubes were centrifuged at 400 g for 10 min. The cells (~0.7×107 cells) that moved to the Percoll solution between the density of 1.033 and 1.047 g/ml were collected. The cells were washed with CMF-PBS and cultured on a 0-1% gelatin-coated dish in MCDB131 medium containing FBS, ECGS, and antibiotics (as above). The medium was changed daily. To check the characterization of luteal ECs, the expression of CD31 (PECAM), an EC marker, was assessed using reverse transcription PCR (RT-PCR) and immunostaining (Fig. 1). The percentage of ECs in the luteal EC preparation that were evaluated by positive staining with anti-CD31 antibody was 89 ± 2-3. The expression of steroidogenic enzyme mRNAs was also determined by RT-PCR. Additionally, western blot analysis for a steroid metabolizing enzyme, 11β-hydroxysteroid dehydrogenase (11β-HSD; Thurton et al. 2003) as a marker of steroidogenic cells, was performed. The concentrations of P4 in the conditioned medium of the preparation
Figure 1 Characterization of luteal endothelial-like cells (ECs) isolated from highly luteinized rat ovaries. (A) Highly luteinized ovaries were collected from eCG- and hCG-treated rats. Whole ovarian dispersed cells were subjected to the centrifugation procedure for endothelial cell enrichment with the gradient Percoll solution, as described in Materials and Methods. The cells that moved to the solution between the density of 1.033 and 1.047 g/ml were collected, and regarded as luteal microvascular ECs (LEC). (B) Whole ovarian cells were cultured for 48 h in MCDB131 medium containing 10% FBS and endothelial growth supplement (ECGS). (a) The cells are shown using phase-contrast microscopy (original magnification, ×100). The trypsinized cell preparation was subjected to the purification of ECs using the gradient Percoll method. The collected cell suspension was seeded on the cover slip in the 24-well dish and cultured for 24 h in the MCDB131 medium (b–d). LEC preparation was stained with anti-CD31 antibody. Immunofluorescence staining (red) of CD31 was detected with Alexa Fluor 594 conjugated with secondary antibody, and DAPI (blue) was used to counterstain (×100; b, ×200: c). Negative control had only the secondary antibody added in the procedure (×200: d). (C) Upper panel: comparison of the basal progesterone (P4) levels in steroidogenic luteal cell preparation (SLC), whole ovarian dispersed cell preparation (WDC) after culture for 48 h in MCDB131 medium containing 10% FBS and ECGS, and LEC preparation. Each cell preparation was incubated for 18 h with serum-free DMEM (SLC) or MCDB (WDC and LEC). Bottom panel: RNA was subjected to semi-quantitative RT-PCR for CD31, Tie-2, and GAPDH. The PCR products were separated in a 1.5% agarose gel containing ethidium bromide and photographed under u.v. transillumination. *P<0.05, **P<0.01 versus SLC. (D) LEC and the remaining cells except LEC (−LEC) collected by the gradient Percoll methods were cultured for 18 h in serum-free MCDB131 medium. Each cell was analyzed by quantitative real-time RT-PCR for CD31, 3β-hydroxysteroid dehydrogenase (3β-HSD), and cholesterol side chain cleavage cytochrome P450 (P450scc). The conditioned media were used for measuring P4 levels. *P<0.05, **P<0.01 versus LEC. (E) The lysate sample (20 μg protein) from each human umbilical vascular endothelial cell (HUVEC), WDC, LEC, and −LEC preparation was subjected to western blot analysis for 11β-hydroxysteroid dehydrogenase (11β-HSD). (F) LEC preparation was seeded on Matrigel and incubated for 20 h in the absence (−) or presence (+) of VEGF (10 ng/ml). Representative photographs are shown.

were measured after 18 h culture. Further, luteal ECs were seeded on Matrigel and incubated for 20 h to observe tube formation. Subcultured cells at one in vitro passage were used in all experiments.

**Isolation and culture of SLCs**

Purified SLCs were collected from the collagenase-digested suspension using the method of Percoll gradient centrifugation, as described previously (Sakurai et al. 2004). The cells were then cultured at 1×10⁶ cells/ml for 24 h in 0.1% gelatin-coated dishes in DMEM (Invitrogen) supplemented with 10% FBS and antibiotics. To test the effects of co-culture of luteal ECs with SLCs or the addition of conditioned medium of SLCs on angiogenesis, SLCs were subsequently incubated for 3 h in serum-free DMEM and then treated for 6 h with 10 ng/ml of VEGF and/or 10 μM of the selective COX-2 inhibitor, NS-398 (which was added 1 h before VEGF treatment). The 3 h serum-free incubation did not result in the reduction of cell numbers or cell viability.

**RT-PCR**

RT-PCR was carried out for VEGF, COX-2, membrane-associated PGE synthase-1, endothelial markers (CD31, Tie-2), and steroidogenic cell markers (3β-hydroxysteroid dehydrogenase and cholesterol side chain cleavage cytochrome P450). Poly (A)⁺ RNA (200 ng) was subjected to RT-PCR using specific primer pairs and the One-step RNA PCR Kit (AMV, Shiga, TaKaRa, Japan), according to the
manipulator's instructions. The sequences of the primers for CD31 were 5'-CTTACCATGCCAGAGGAGAC-3' (sense; S) and 5'-CCTGCGTATGATGTC TCTGGTG-3' (antisense; AS). The sequences of the primers for Tie-2 were 5'-GGGAAACTGAGAGGACGCAC-3' (S) and 5'-GCATCATCGGATGCACCATC-3' (AS). The predicted lengths of the fragments were 360 bp for CD31 and 516 bp for Tie-2. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences for GAPDH were 5'-ACCACGATCCATGCCCAC-3' (S) and 5'-TCCACCCACCTGTGCTGTA-3' (AS), and the expected size of the amplified product was 452 bp. The PCR protocols used were as follows: 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s (Arosh et al. 2004). The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and were visualized with u.v. light. In addition, the total RNA (100 ng) was subjected to real-time RT-PCR using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories). The reactions were carried out on an q5 Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used for real-time RT-PCR were as follows: 5'-GGAGGAGATCTGGGCCACCTGAC-3' (S), 5'-TGTAGGGCATGGTGGAGACG-3' (AS) for cholesterol side chain cleavage cytochrome P450 (P450scc); 5'-GACCAGAACACAGGAG-3' (S), 5'-CTGGCCAGCTCTCTCAG-3' (AS) for 3β-hydroxysteroid dehydrogenase (3β-HSD); 5'-CCCAGTGCATCTTACAGACA-3' (S), 5'-ACCTTGACCCTCAGATCTC-3' (AS) for COX-2; 5'-AGCCACATGAGGAC-3' (S), 5'-GCCCCATAGCCACATC-3' (AS) for CD31; 5'-AGCCACATGCACATC-3' (AS) for VEGF; 5'-GCTACTGCCATGACC-3' (S), 5'-GGAGGAACACAGAGGAGAC-3' (S), 5'-CTGGCCAGCTCTCTCAG-3' (AS) for GAPDH. The fold change in expression of each gene was calculated using the ΔΔCt method with GAPDH as an internal control.

Immunostaining of CD31
To examine the expression of CD31 in cultured cells, immunofluorescence staining was performed. Cells were plated at 2×10^4 cells per well onto poly-l-lysine-coated cover slips (Asahi Techno Glass Co., Tokyo, Japan) in 24-well dishes. Cells fixed with 4% (w/v) paraformaldehyde (PFA) were treated with PBS containing 0.5% (w/v) Triton X-100 at room temperature (R/T) for 5 min for permeabilizing cell membrane, blocked overnight with 3% (w/v) BSA in PBS (PBSB), and incubated with 5 μg/ml anti-rat CD31 antibody (US Biological, Inc., Minneapolis, MN, USA) in PBS containing 1% (w/v) BSA for 1.5 h. After washing with PBS containing 0.1% Tween-20 (PBST), the cells were incubated with secondary antibody, Alexa Fluor 594-labeled goat ant-mouse IgG (10 μg/ml). For negative control staining, cells were incubated with mouse IgG instead of primary antibody. Nuclear counter staining was performed using 300 nM of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen). The cells were mounted and sealed using SlowFade gold antifade reagent (Invitrogen). After washing with PBST, the cells were incubated with 2 μg/ml anti-rabbit IgG antibody conjugated with HRP. The slips were developed with PBS containing 1-4 mM of 3,3′-diaminobenzidine with 0.03% H2O2 after washing.

Measurement of P4 and PGE2
The concentration of P4 in the cultured medium was measured by RIA, as described previously (Sakurai et al. 2004). The levels of PGE2 in the culture media were determined using the PGE2 Enzyme Immunoassay (EIA) Kit (Assay Designs, Inc., Ann Arbor, MI, USA), according to the manufacturer’s instructions.

In vitro endothelial tubular formation on Matrigel
The tube formation assays were performed using 48-well dishes coated with 100 μl of Matrigel per well, which was allowed to solidify at 37°C for 30 min. Luteal ECs that had been preincubated for 3 h with serum- and ECGS-free MCD131 medium were resuspended at a density of 2×10^5 cells/ml in the MCD131 medium. The cell suspension was then plated at 500 μl onto the surface of the Matrigel and incubated under various conditions. After 24 h, the cells were fixed in 4% PFA and photographed at ×4 magnification with a digital camera attached to an inverted microscope. The measurement was done on three randomly chosen microscopic fields per well, and the mean of the three fields was used as a single observation. Tube formation was quantified by measuring the number of junctions or joint forming cell–cell networks and of the total length of tube-like cells using the KSW-5000U software (Kurabo, Tokyo, Japan; Tamura et al. 2006). Experiments that were performed for the analysis of tubular formation were repeated at least three times.

Immunoblot analysis
Cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology, Beverly, MA, USA). The cell lysates (5 μg protein) were subjected to 10–20% gradient SDS-PAGE, and blotting membranes were prepared. The membranes were blocked with Immunoblock (Dainippon Sumitomo Pharma Co. Ltd, Tokyo, Japan) and then incubated with each primary antibody (1:1000 dilution), i.e. anti-phospho extracellular signal-regulated kinase (ERK) antibody, anti-phospho AKT antibody, anti-ERK antibody, anti-phospho AKT antibody (0.1% (v/v) antibody, anti-ERK antibody, anti-phospho AKT antibody (1:1000 dilution), i.e. anti-phospho extracellular signal-regulated kinase (ERK) antibody, anti-phospho AKT antibody, anti-ERK antibody, anti-phospho AKT antibody (0.1% (v/v) each; Cell Signaling Technology), or 11β-HSD (type I) antibody (Cayman Chemical) in Tris–buffered saline containing 0.1% (v/v) Tween-20 (TBST). The membranes were subsequently washed in TBST. The immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Science, Wellesley, MA, USA) after incubation with HRP-labeled mouse or rabbit IgG antibody.
(0.5 μg/ml; Vector Laboratories, Burlingame, CA, USA). All blotting experiments were repeated at least three times, and representative data are shown.

Statistical analysis

Data were statistically analyzed by Student’s t-test or one-way ANOVA when comparing more than two categories followed by Tukey’s multiple comparison test. Results are expressed as mean ± s.e.m. Differences were considered statistically significant when P < 0.05.

Results

Characterization of isolated luteal ECs

The luteal EC preparation moved between the density of 1.033 and 1.047 g/ml in the Percoll solution and was isolated from total luteinized ovarian cells cultured in MCDB medium, as shown in Fig. 1A. The average cell population was 18% of total cells applied. The immunocytochemical signal of CD31 was observed in cultured EC preparation (Fig. 1B), with the percentage of CD31-negative cells being ~10%. The preparations of SLCs, whole ovarian cells before the purification (WDC), and ECs (LEC in Fig. 1) were cultured for 18 h in serum-free MCDB131 medium (Fig. 1C). The EC preparation exhibited low capacity of P₄ production, and the levels were significantly lower than those in SLC. The expression of CD31 and Tie-2 mRNA was semiquantitatively higher in luteal ECs than that in SLC and WDC. Furthermore, luteal ECs and the remaining cells except luteal ECs (−LEC in Fig. 1) after separation by the gradient Percoll methods were quantitatively compared by real-time RT-PCR for P450scc, 3β-HSD, and CD31 (Fig. 1D). The levels of P450scc and 3β-HSD in luteal ECs were significantly lower than those of −LEC, whereas the CD31 levels were higher than −LEC. The levels of P₄ in the conditioned media were obviously lower in LEC than in −LEC (Fig. 1D). Western blot analysis for 11β-HSD (Fig. 1E) has revealed that the luteal EC preparation did not show a clear band of 11β-HSD, whereas both WDC and −LEC preparations exhibited distinct bands of the enzyme. As shown in Fig. 1E, the tubular formation in the EC preparation was stimulated by the addition of VEGF (10 ng/ml), whereas SLC did not show distinct tubular formation on Matrigel (data not shown). Thus, the luteal EC preparation formed tubular morphology that is a characteristic of vascular ECs. Based upon these results, we regarded this preparation as enriched luteal microvascular ECs derived from highly luteinizing rat ovary.

Effects of COX inhibitors on VEGF- and/or PGE₂-stimulated tubular formation in luteal ECs

Figure 2 shows the effects of VEGF and/or PGE₂ in the presence or absence of COX inhibitors (SC–560, COX–1 inhibitor; NS–398, COX–2 inhibitor) on tube formation in luteal ECs. SC–560 or NS–398 at the dose of 10 μM, which is enough to prevent endogenous PGE₂ production (Sakurai et al. 2004), significantly suppressed basal levels of tube formation. PGE₂ treatment promoted tube formation, even in the presence of SC–560 or NS–398. The VEGF-stimulated tube formation was also reduced by SC–560 or NS–398 treatment. Both treatment with PGE₂ and VEGF (COX inhibitor: −/PGE₂+VEGF) increased tube formation when compared with treatment with PGE₂ or VEGF alone; however, these enhanced levels were reduced by incubation with either SC–560 or NS–398 to the levels of the group treated with PGE₂ alone.

Effect of the EP receptor antagonists on PGE₂-enhanced tube formation and db-cAMP on tube formation in luteal ECs

The effect of the EP receptor antagonists on the PGE₂-stimulated tube formation was examined (Fig. 3A and D). Each antagonist alone did not significantly decrease the basal levels of tube formation (data not shown). PGE₂-dependent increase in tube formation was completely blocked by the EP2 antagonist. A partial inhibitory effect by the EP4 receptor antagonist was observed. No effect in the extent of tube

Figure 2 Effects of COX inhibitors on PGE₂- and/or VEGF-stimulated tubular formation in luteal ECs. Cells were seeded on Matrigel and treated for 20 h in the presence or absence of PGE₂ (1 μM; 350 ng/ml) and/or VEGF (10 ng/ml) with or without SC560 (SC: 10 μM, selective COX-1 inhibitor) or NS-398 (NS: 10 μM, selective COX-2 inhibitor). The representative photographs of tube formation with or without NS-398 are shown in the upper panel. Tube formation was determined by the assessment of the total length of tubes in three randomly selected fields. Data are expressed as the ratio of the control value. Each value represents the mean ± s.e.m. for three separate experiments. *P<0·05, **P<0·01 versus control.
formation was observed in cells treated with the EP1 receptor antagonist. cAMP serves as a second messenger via the EP2 and EP4 receptors. db-cAMP, which is a stable cAMP analog, stimulated tube formation at a concentration of 100 or 1000 μM (Fig. 3B). Furthermore, the EP2 receptor antagonist suppressed partially, but significantly, VEGF-stimulated tube formation (Fig. 3C). Figure 3D shows the representative picture of tube formation in each treatment. Additional experiment has shown that H89, a PKA inhibitor, clearly blocked PGE2-induced tube formation as well as forskolin (an adenylyl cyclase activator)-induced changes.

Effects of PGE2 and the EP receptor antagonist on phosphorylation of ERK and Akt in luteal ECs

In addition to activation of cAMP signaling pathway, the involvement of different signaling pathways via the EP2 receptor on tube formation was examined (Fig. 4), because there are some evidences showing that the PGE2-mediated pathways include the activation of MAPK/ERK and AKT/GSK3β (Regan 2003, Yu et al. 2008). Cells were treated with both the EP1 and EP2 receptor antagonists or both the EP1 and EP4 receptor antagonists, because the EP1 receptor antagonist did not give significant influence on the levels of phosphorylation of AKT and ERK. As shown in Fig. 4, PGE2 increased the phosphorylation of both ERK and AKT with intact EP1, 2, 3, and 4 receptors. However, the PGE2-stimulated ERK and AKT phosphorylations were suppressed in the presence of both the EP1 and EP4 receptor antagonists, whereas these phosphorylations were observed under the treatment with the EP1 and EP2 receptor antagonists.

Effects of PGE2 on VEGF expression and of VEGF on PGE2 production in luteal ECs

The effect of PGE2 on the VEGF mRNA expression in luteal ECs was examined (Fig. 5A and B: left). PGE2 (0.3, 1, and 1000 μM) before seeding on Matrigel and were then treated with PGE2 (1 μM). Each value represents the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 versus control. (B) Cells were treated with PGE2 (1 μM) or db-cAMP (10–1000 μM). Each value represents the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 versus control. (C) Cells were treated for 1 h with both SC19220 (EP1 receptor antagonist; 10 μM), AH6809 (EP2 receptor antagonist; 10 μM), or AH23848 (EP4 receptor antagonist; 10 μM) before seeding on Matrigel and were then treated with PGE2 (1 μM). Each value represents the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 versus control. (D) The representative photographs of tube formation with changes in each treatment are shown. PGE2/EP2 or EP4:PGE2 treatment in the presence of EP2 or EP4 receptor antagonist, Fors; Forskolin.

Figure 3 Effects of the prostanoid EP receptor antagonist on PGE2- or VEGF-stimulated tube formation and of db-cAMP on tube formation in luteal ECs. Luteal ECs were cultured for 20 h on Matrigel in a 24-well plate. Tubular formation was measured by the total length of tubes in three randomly selected fields, and the data are expressed as a ratio of the control value. (A) Cells were pre-treated for 1 h with SC19220 (EP1 receptor antagonist; 10 μM), AH6809 (EP2 receptor antagonist; 10 μM), or AH23848 (EP4 receptor antagonist; 10 μM) before seeding on Matrigel and were then treated with PGE2 (1 μM). Each value represents the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 versus control. (B) Cells were treated with PGE2 (1 μM) or db-cAMP (10–1000 μM). Each value represents the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 versus control. (C) Cells were pre-treated for 1 h with the EP2 or EP4 receptor antagonist (AH6809 or AH23848; 10 μM), and were then treated with VEGF (1 μM). Each value represents the mean ± S.E.M. of three experiments. (D) The representative photographs of tube formation in each treatment. Additional experiment has shown that H89, a PKA inhibitor, clearly blocked PGE2-induced tube formation as well as forskolin (an adenylyl cyclase activator)-induced changes.

Figure 4 Effects of PGE2 and EP receptor antagonists on phosphorylation of ERK and Akt in luteal ECs. Cells were pre-incubated for 1 h with both SC19220 (EP1 receptor antagonist; 10 μM) and AH6809 (EP2 receptor antagonist; 10 μM), or both SC19220 and AH23848 (EP4 receptor antagonist; 10 μM), and were then treated for 20 min with PGE2 (1 μM). The cell lysates were subjected to SDS-PAGE and western blot analysis using each antibody. The activation of ERK1/2 and AKT was evaluated by immunoblot analysis using phospho-ERK1/2 and AKT antibodies respectively. To ensure that equal amounts of protein were analyzed, total ERK and AKT levels were determined in cell lysates.
3 μM) stimulated the expression of VEGF mRNA. DB-cAMP (10–1,000 μM) also enhanced the VEGF level in a dose-dependent manner (data not shown). Our previous report has demonstrated that VEGF stimulates PGE2 secretion in SLCs (Sakurai et al. 2004). To determine the influence of VEGF on PGE2 production in luteal ECs, the cells were treated with various doses of VEGF (Fig. 5A and B: right panels). Quantitative RT-PCR data showed that VEGF increased COX-2 mRNA levels 2 h after treatment in a dose-dependent manner (0.3–3 ng/ml; Fig. 5B). The mRNA levels of membrane-associated PGE synthase-1 (mPGES-1) tended to be increased by VEGF (3–30 ng/ml) but were not statistically significant. The concentration of PGE2 in the culture medium was determined (Fig. 5C); VEGF increased PGE2 secretion, and significant upregulation was detected at 10 and 30 ng/ml of VEGF.

**Figure 5** Effect of PGE2 on VEGF expression and of VEGF on PGE2 synthesis in luteal ECs. (A) Cells were treated for 2 and 6 h with different doses of PGE2 (0.03–3 μM; left panel) and VEGF (0–1–30 ng/ml; right panel) respectively. The RNA extracted from samples was subjected to semi-quantitative RT-PCR analyses to determine each mRNA expression levels. GAPDH was used as an internal control. Representative data are shown. mPGES-1; membrane-associated PGE synthase-1. (B) Cells were treated with PGE2 or VEGF, as described earlier. RNA was subjected to quantitative RT-PCR analysis to determine mRNA levels of VEGF (left panel) and COX-2 (right panel). The data from three independent experiments are shown. *P<0.05, **P<0.01 versus control. (C) Cells were incubated for 24 h with VEGF, and the PGE2 concentration in the media was measured using EIA. Each value represents the mean ± S.E.M. of three cultures. **P<0.01 versus control.

**Effect of co-culture with SLCs on tube formation in luteal ECs**

To investigate the impact of SLCs and the significance of the EP2 receptor on tube formation in luteal ECs, the effect of the co-culture of luteal ECs with SLCs was examined by using chemotaxicell, as shown in Fig. 6A. SLCs were incubated for 6 h with VEGF (10 ng/ml) and/or NS-398 (10 μM) (b, c, d in Fig. 6A) and co-cultured for 20 h with luteal ECs in the presence or absence of the EP2 receptor antagonist. Co-culture with SLCs enhanced the tube formation in luteal ECs (a in Fig. 6B), and the stimulatory effect of co-culture was further increased when SLCs were pretreated with VEGF (b in Fig. 6B); however, this effect was inhibited by the incubation of cells with NS-398 (c in Fig. 6B). Treatment with the EP2 receptor antagonist significantly abolished the stimulatory effect of SLC co-culture on tube formation. The quantification of the concentration of PGE2 in the medium after 20 h of culture (Fig. 6C; SLCs versus a) revealed a significant increase in PGE2 in the co-culture system. An increase of approximately twofold was observed in the VEGF-treated group (a versus b), whereas low PGE2 levels were observed in the NS-398-treated group (c and d). However, the EP2 receptor antagonist did not affect co-culture- and/or VEGF-enhanced PGE2 production.

**Discussion**

This study was undertaken to isolate rat luteal microvascular ECs from highly luteinized ovaries in order to demonstrate the involvement of PGE2 in the functions of ECs. The EC preparation expressing CD31 showed low capacity of P4 production, compared with the remaining preparation that excluded the EC fraction. P450scc is responsible for the first step in steroidogenesis, catalyzing the conversion of cholesterol to pregnenolone (Tuckey 2005). The expression of 3β-HSD, a key enzyme for the synthesis of P4 through pregnenolone, profoundly affects the level of P4 secreted by the CL (Tuckey 2005). Two types of 11β-HSD isoforms are present in granulosa-lutein cells, and both enzymes may be upregulated by LH during luteinization (Thurton et al. 2003, Myers et al. 2008). The expressions of P450scc, 3β-HSD, and 11β-HSD in the luteal EC preparation were considerably lower than those in the total ovarian cell preparation that excluded ECs. The cultured ECs showed tubular morphology responded to VEGF. Thus, we have successfully isolated the EC-enriched preparation showing the typical characteristics of vascular ECs. Interestingly, some granulosa cells have been shown to exhibit characteristics of ECs (Antczak & Van Blerkom 2000). The multipotency of granulosa stem-like cells has been described by in vivo differentiation into other cell types (Kossowska-Tomaszcuk et al. 2009). In vitro culture of glioblastoma stem-like cells in EC condition generated progeny with phenotypic and functional features of ECs (Ricci-Vitiani et al. 2010). The EC preparation in this study might include a subset of
granulosa stem-like cells that show characteristics of endothelial progenitors cells of maturation into ECs under endothelium condition. This study is, to the best of our knowledge, the first to show in vitro tube formation in rat luteal ECs, although luteal ECs have already been isolated and characterized from other species including cows (Okuda et al. 1999, Davis et al. 2003), rhesus monkeys (Christenson & Stouffer 1996), and humans (Ratchiffe et al. 1999).

The PGE2-induced tube formation was observed either in the presence of COX inhibitors or in the presence of a blocker of the VEGF receptor-2, and without EC proliferation (data not shown). Addition of PGE2 stimulated in vitro tube formation of luteal ECs, which was completely attenuated by treatment with an EP2 receptor antagonist. It has been shown that the EP2 receptor activates an intracellular signal, primarily through cAMP-dependent protein kinase A (PKA), which means that cAMP serves as a second messenger of the receptor (Bos et al. 2004). In this study, the inhibitor of PKA, H89, completely blocked PGE2-induced tube formation and db-cAMP, which is a stable cAMP analog, enhanced tube formation in a dose–dependent manner. Activation of the AKT and MAP kinases induced by PGE2 was inhibited by EP4 antagonist, but not by EP2 antagonist. Thus, PGE2-induced tube formation of luteal ECs is probably mediated through the EP2-dependent cAMP signaling pathway, and the PGE2/EP2-mediated pathway is not as closely linked to the MAPK/ERK and AKT pathways as the PGE2/EP4-mediated pathway in luteal ECs. These findings were consistent with a review (Regan 2003) describing the differential signaling of the EP2 and EP4 receptors. The EP2 and EP4 receptors have already been suggested to play a role in PGE2-regulated vascular function (Bos et al. 2004). The piglet saphenous vein expresses the EP2 and/or EP4 receptors, which are coupled to smooth muscle relaxation (Wilson & Giles 2005). The EP2 receptor was demonstrated to contribute directly to EC migration and survival (Kamiyama et al. 2006), and cytoskeletal remodeling partially mediated through Rac-GTPase (Birukova et al. 2007) in ECs. The PGE2/EP2-induced signal transduction might modulate the state of endothelial cytoskeleton to influence tube formation. The EP2 receptor is highly expressed in human CL (Narko et al. 2001) and associated with cortisol metabolism via 11β-HSD in human granulosa-lutein cells (Chandras et al. 2007), and cytoskeletal remodeling partially mediated through Rac-GTPase (Birukova et al. 2007) in ECs. The PGE2/EP2-induced signal transduction might modulate the state of endothelial cytoskeleton to influence tube formation. The EP2 receptor is highly expressed in human CL (Narko et al. 2001) and associated with cortisol metabolism via 11β-HSD in human granulosa-lutein cells (Chandras et al. 2007), and cytoskeletal remodeling partially mediated through Rac-GTPase (Birukova et al. 2007) in ECs. The PGE2/EP2-induced signal transduction might modulate the state of endothelial cytoskeleton to influence tube formation.

Our results suggest that the EP2 receptor may play a critical role in PGE2-dependent tube formation in rat CL. Furthermore, a recent study has shown that the developing rhesus CL has the highest levels of the EP3 receptor (Bogan et al. 2008). PGE2 signaling via the EP3 receptor has been implicated in tumor-associated angiogenesis (Taniguchi et al. 2008, Amano et al. 2009). We did not mention the involvement of the EP3 receptor in this study, because the EP3 antagonist was not available. However, our observation showing no significant effect of sulprostone, an EP3 agonist on tubular formation in the presence of COX-2 inhibitor (data not shown), probably indicates that the EP3 receptor may not be associated with rat luteal EC functions.

In addition, PGE2 suppresses apoptosis of several cell types including bovine SLCs (Bowolaksono et al. 2008), rat monocyte/macrophage (Tommasini et al. 2008), and human endometriotic cells (Banu et al. 2009). We cannot exclude the
possibility that the anti-apoptotic effect of PGE₂ on luteal ECs or SLCs might affect cellular activity during the 20 h culture for inducing the formation of tubules. Thus, the signaling pathway and cellular response elicited by PGE₂ are complicated and this seems to be dependent upon the difference of the cell types and/or species. The results suggest that PGE₂ may promote angiogenesis in newly formed CL, although the experimental in vitro approach did not exactly study angiogenesis, and it is difficult to conclude that the doses of PGE₂ applied in this study are physiological levels locally produced in the ovulated follicles.

Our previous data showed that ovarian angiogenesis in the early luteal phase was suppressed by COX-2 inhibitors (Sakurai et al. 2003), and that its inhibition was restored by i.u. injection of PGE₂ (Sakurai et al. 2005). We have therefore hypothesized that suppression of luteal angiogenesis in COX-2 inhibitor-treated rats may be due to a decrease in the stimulatory effect of PGE₂ on angiogenesis (Sakurai et al. 2003), which is probably caused by the reduction in PGE₂ secretion in the CL. In the ovary, follicular PGE₂ concentrations gradually increase during the follicular phase, and PGE₂ levels are markedly elevated in mature granulosa cells after the LH surge and remain high during the development of the newly formed CL (Brown & Poyser 1984). Considering that luteinized granulosa cell is a major component in early CL and secretes PGE₂, we can suppose that SLC-derived secreted PGE₂ might contribute to luteal angiogenesis during the early stage of CL formation in a paracrine fashion in vivo, because the examination of the impact of co-culture with SLCs revealed that the factor(s) that was secreted from SLCs and was inhibited by the COX-2 inhibitor promoted angiogenesis (Fig. 6). The conditioned medium of the co-culture system that enhanced EC tube formation possessed clearly high levels of PGE₂, and the stimulatory effect was also completely blocked by treatment with an EP2 receptor antagonist. Furthermore, conditioned medium of only SLC preparation promoted tube formation of luteal ECs, and the stimulation was attenuated by an EP2 receptor antagonist (data not shown). This supports the possibility that PGE₂, which is secreted from SLCs, may stimulate tube formation of ECs in CL.

VEGF is essential for the development of the vasculature (Shweiki et al. 1993, Ferrara & Davis-Smyth 1997, Pauli et al. 2005). Several reports suggest that the stimulatory effect of PGE₂ on angiogenesis is mediated by VEGF production in ECs (Nie & Honn 2004, Lopes et al. 2006). In this study, we showed that PGE₂ treatment increased VEGF expression in luteal ECs. In rat SLCs (Sakurai et al. 2004) as well as in porcine SLCs (Kowalczyk et al. 2008), PGE₂ enhanced VEGF mRNA expression, and in turn VEGF-stimulated COX-2 and mPGES in rat SLCs (Sakurai et al. 2004). The COX-2 inhibitor suppressed VEGF expression and PGE₂ production in SLCs (Sakurai et al. 2004). It has been reported that the VEGF-mediated elevation of COX-1 expression may be associated with angiogenesis via the maintenance of vascular integrity in aortic ECs (Bryant et al. 1998), and that the VEGF-mediated induction of COX expression may promote the proliferation of vascular cells and prostate cancer (Mukherjee et al. 2005). Thus, COX expression seems to profoundly affect angiogenesis of ECs. ECs derived from EP2 knockout mice show lower responsiveness to VEGF when compared with normal mice (Chang et al. 2005). In this study, VEGF induced COX-2 expression, as well as PGE₂ secretion in luteal ECs. COX-2-induced PGE₂ is probably important for the function of luteal ECs in vivo, because COX-1 inhibitor had no effect on the formation of CL (Sakurai et al. 2003) and VEGF did not influence COX-1 expression (Fig. 5). These findings suggest that VEGF actions might be partially dependent on COX-2-mediated products in ECs, as has been discussed in our previous reports (Sakurai et al. 2004, Tamura et al. 2006). We observed that treatment with an EP2 receptor antagonist causes a significant decrease in VEGF-enhanced tube formation. This suggests that the EP2 receptor-mediated signal promotes VEGF action. VEGF-stimulated tube formation might be partially mediated through EP2 receptor stimulation induced by increases in PGE₂ secretion. Recently, the synergistic interaction between PGE₂ and fibroblast growth factor (FGF)-2 signaling pathways has been described (Finetti et al. 2009). PGE₂ induces endothelial activation by upregulating the FGF-2 pathway. Luteal PGE₂ might be involved in formation of new capillary vessels acting in cooperation with other well-known angiogenic factors when the ruptured follicles initiate distinct vascularization. Thus, it seems likely that the angiogenic effect of VEGF on luteal ECs in vivo includes both a direct action mediated by the VEGF receptor and an indirect action on SLCs and ECs mediated by PGE₂ production.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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