Roles of steroid hormones in bovine endometrial prostaglandin synthesis

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PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Environmental and Life Science (Doctor Course), Okayama University, Japan, from April 2013 to September 2015, under the supervision of Professor Kiyoshi OKUDA.

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

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ABSTRACT

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In ruminants, the corpus luteum is a transient endocrine gland that is formed following ovulation. The main function of the corpus luteum is secretion of progesterone (P4), which is essential for establishment and maintenance of pregnancies. As long as the corpus luteum persists, P4 secreted by the ovary feeds back to the hypothalamus and pituitary to interrupt the estrous cycle. Thus, in non-pregnant cows, the corpus luteum must be eliminated from the ovary to permit a new ovarian cycle. Prostaglandins (PGs) are mediators of inflammatory response and smooth muscle contraction. In non-pregnant cows, pulsatile release of PGF2α (PGF) from the endometrium occurs between days 17 and 19 post-ovulation. Uterine PGF regulates the estrous cycle by inducing luteolysis. Conceptus alters the ratio of PGE2 to PGF in the uterine vein, and PGE2 continuously stimulates luteal function to maintain pregnancy.

Steroid hormones play important roles as endometrial PG production regulatory factors. In some animals, P4 suppresses oxytocin (OT) receptor expression in the endometrium to decrease PGF production stimulated by OT. Furthermore, active glucocorticoid (cortisol: Cr) has been demonstrated to suppress PGF production in cultured bovine endometrial stromal cells but not epithelial cells. However, details of the PGF production regulatory mechanisms of these steroid hormones remain unclear. In the present study, to clarify the controlling mechanisms of endometrial PG production, we investigated 1) the possible roles and actuating pathways of P4 on PG synthesis in cultured bovine endometrial epithelial and stromal cells and 2) the suppressive effect of Cr on PGF production by cultured endometrial tissues during the estrous cycle.

(1) To investigate the effect of P4 via nuclear P4 receptor on PG synthesis, cultured endometrial cells were treated with P4 (1, 10, or 100 nM) for 24 h. PGF and PGE2 in cultured media and mRNA expression of PG synthase (phospholipase A2, PLA2; cyclooxygenase-2, COX-2) were examined by enzyme immunoassay (EIA) and quantitative RT-PCR, respectively. To clarify whether *PLA2* and *COX2* mRNA expression was influenced by P4 in a very short time, endometrial epithelial and stromal cells were pre-incubated with fresh medium for 1 h. After incubation, the medium was replaced with fresh medium, and cells were exposed to P4 (0 or 10 nM) for 5, 10, 15, or 20 min. Then, *PLA2* and *COX2* mRNA expression was examined by quantitative RT-PCR. To reveal the effect of membrane-mediated P4 on PGF and PGE2 production,

cultured endometrial cells were treated BSA-conjugated P4 (P4-BSA; 1, 10, or 100 nM) for 24 h. P4-BSA specifically binds to cell surface membranes. PGF and PGE2 concentrations in the media of both cell types were measured by EIA. Finally, the expression of *PGR*, *PAQR7*, *PGRMC1*, *PGRMC2*, and *SERBP1* mRNA in endometrial tissues of each stage (n=5 per stage) were examined by quantitative RT-PCR.

Progesterone treatment for 24 h stimulated PGE2 production in epithelial cells ($P<0.05$) but suppressed both PG production ($P<0.05$) and the expression of PG-metabolizing enzymes, including phospholipase A2 (PLA2) and cyclooxygenase-2 (COX2) in stromal cells ($P<0.05$). Short-term P4 treatment (5–20 min) did not affect *PLA2* or *COX2* transcript levels in either cell type. P4-BSA only increased PGF and PGE2 production in epithelial cells ($P<0.05$). Nuclear P4 receptor mRNA expression in the endometrium was higher during the follicular phase than during the early- to mid-luteal stages ($P<0.05$), whereas membrane P4 receptor mRNA expression did not change throughout the estrous cycle. These results indicate that P4 has different effects on PG production by inhibiting PG synthase and stimulating signal transduction via a membrane P4 receptor. Consequently, P4 may protect the corpus luteum by attenuating PGF production in stromal cells and increasing PGE2 secretion by epithelial cells.

(2) To investigate the suppressive effect of Cr on PGF production in bovine endometrium throughout the estrous cycle, the protein expression of glucocorticoid receptor α (GC-Rα) in endometrial tissues of each stage (n=3 per stage) was examined by western blotting. Then, to determine the relationship between receptor expression and Cr interaction, endometrial tissues obtained during the mid-luteal stage and follicular phase were treated with Cr (10 nM) for 4 h. The concentrations of PGF in the culture media after incubation were determined by EIA. Cultured endometrial stromal cells were treated with Cr (1, 10, or 100 nM), P4 (0.1, 1, or 10 nM), or E2 (0.1, 1, or 10 nM) for 24 h. After incubation, GC-Rα mRNA and protein expression was determined by quantitative RT-PCR and western blotting.

GC-Rα protein expression was higher during the mid- and late-luteal stages than during the other stages ($P<0.05$). Cr (10 nM) decreased PGF production in endometrial tissue collected during the mid-luteal stage ($P<0.05$), but not in endometrial tissue collected during the follicular stage. Cr decreased GC-Rα mRNA and protein expression, and the effect was significant at 100 nM in cultured bovine endometrial stromal cells ($P<0.05$). P4 increased GC-Rα mRNA and protein expression, and the effect was significant at 10 nM in cultured bovine endometrial stromal cells ($P<0.05$). Exposing cultured bovine endometrial stromal cells to E2 did not affect *GC-Rα* mRNA expression but significantly decreased GC-Rα protein expression ($P<0.05$). The findings of this study indicate that the expression of GC-Rα is correlated with the suppressive effect of Cr on endometrial PGF production, especially during the mid-luteal stage, and the expression is up-regulated by P4.

Overall, the results herein indicate that steroid hormones control endometrial PG production to determine the fate of the corpus luteum.

CHAPTER 1

GENERAL INTRODUCTION

Reproductive dynamics during the estrous cycle

Morphological and physiological changes throughout the estrous cycle in female reproductive organs

The estrous cycle (ovulatory cycle) in cattle lasts for 18–24 days [1]. In heifers, the onset of estrous cycles, which is equivalent to puberty, occurs at 6–12 months of age [1]. During the estrous cycle, there are various phenomena, including follicle growth, ovulation, luteinization, and luteolysis in the ovary. The estrous cycle consists of two discrete phases: the luteal phase (14–18 days) and the follicular phase (4–6 days) [1].

Ovarian functions are regulated by endocrine hormones secreted by the hypothalamus (gonadotrophin-releasing hormone, GnRH), anterior pituitary (follicular stimulating hormone, FSH; luteinizing hormone, LH), ovaries (progesterone, P4; estradiol-17β, E2; and inhibin), and uterus (prostaglandin F2α, PGF) [2]. During the follicular phase of the estrous cycle, follicles develop in the functional ovaries; then, follicular growth coincides with selection of the dominant follicle [1]. These waves of ovarian follicle growth are correlated with FSH secretion from the anterior pituitary, which generally occur two (dairy cows) or three times (heifers and beef cows) during the cycle [1]. As a result of pulsatile secretion of LH, oocytes are released by mature follicles and released into the oviduct, allowing the potential for fertilization [1]. Following ovulation, the concentration of P4 in blood plasma begins to increase because of the formation of the corpus luteum, in which the granulose and theca cells of the ovulated dominant follicle differentiate into luteal cells [3]. The luteal cells produce P4 to prepare for the establishment and maintenance of pregnancy and/or resumption of the estrous cycle [3]. In non-pregnant cows, the uterus increases PGF production between days 17 and 19 post-ovulation [4]. The increase in uterine PGF production and its pulsatile release are responsible for luteolysis and initiation of a new estrous cycle in mammals [5-7].

Biosynthetic pathway and biological action of PGs in the endometrium

In general, the first step involved in prostaglandin formation is the hydrolytic release of arachidonic acid, which is mediated by members of the phospholipase A2 (PLA2) family of enzymes [8]. Following its release, arachidonic acid is converted to PGH2 by cyclooxygenase-2 (COX2), which is situated on the luminal surface of the endoplasmic reticulum [9]. After biosynthesis of PGH2, both PGF and PGE2 are synthesized from PGH2 by two specific downstream enzymes, PGH 9, 11-endoperoxide reductase (also known as prostaglandin F synthase; PGFS), and prostaglandin E synthase (PGES) [10]. A small amount of PGF is also produced from PGE2 by PGE2 9-ketoreductase [11, 12].

From days 6–17 of the estrous cycle, the endometrial environment is dominated by P4, which is secreted by the ovary [13]. Following that, E2 secreted by follicles induces oxytocin release, and PGF production increases in the endometrial epithelial cells to induce luteolysis [13]. In contrast to PGF, PGE2 has luteoprotective properties [14, 15]. PGE2 is also required for changes in vascular permeability that occur in the endometrium

before implantation in rodents [16]. Interferon-τ, which is of trophoblast origin during the pre-implantation period, has been demonstrated to stimulate PGE2 production and *COX-2* expression in the bovine endometrium in vitro [17]. In bovine, interferon-τ inhibits the E2 receptor, which is necessary for induction of the oxytocin receptor before luteolysis [18].

The aim of the present study

To clarify the controlling mechanisms of endometrial PG production, I investigated 1) the possible roles and the actuating pathways of P4 on PG synthesis in cultured bovine endometrial epithelial and stromal cells and 2) the suppressive effect of active glucocorticoid hormone (cortisol: Cr) on PGF production by endometrial tissue throughout the estrous cycle.

CHAPTER 2

GENERAL METHODOLOGY

Collection of endometrial tissues

Apparently-healthy uteri of Holstein cows without a visible conceptus were obtained from a local slaughterhouse (Okayama Meat Center) within 10–20 min of exsanguination and were immediately transported to the laboratory. The stages of the oestrous cycle were confirmed by macroscopic observation of the ovaries and uterus as described previously [19, 20]. For mRNA analysis, endometrial tissues (n = 5 per stage) were collected from cows at six different stages of the oestrous cycle (oestrus, Day 0; early-luteal, Days 2–3; developing, Days 5–6; mid-luteal, Days 8–12; late-luteal, Days 15–17 and follicular stage, Days 19–21). Intercaruncular endometrial tissues from the uterine horn, ipsilateral to the CL or the dominant follicle, were used for experiments. The endometrial tissues were immediately frozen in liquid nitrogen and stored at –80°C until processed for mRNA and protein isolation. For experiments involving tissue culture and cell culture, the uterus were submerged in ice-cold physiological saline and transported to the laboratory within 1-1.5 h on ice.

Isolation of endometrial cells

Uteri of the early and developing luteal stages (Days 2–3 and 5–6) were used for isolation. The epithelial and stromal cells from bovine endometrium were enzymatically separated (0.05% collagenase; Worthington Biochemical Co., Lakewood, NJ, USA) using procedures described previously [21]. A polyvinyl catheter was inserted into the side of the oviduct and the ends of the horn were tied to retain trypsin solution used to detach the epithelial cells as described herein. The uterine lumen was washed three times with 30–50 mL of sterile Ca^{2+}-free and Mg^{2+}-free Hank's balanced salt solution (HBSS) supplemented with 100 IU mL^{-1} penicillin (Meiji Seika Pharma, Tokyo, Japan), 100 µg mL^{-1} streptomycin (Meiji Seika Pharma) and 0.1% (w/v) bovine serum albumin (BSA) (Roche Diagnostics, Manheim, Germany). Thirty to fifty millilitres of sterile HBSS containing 0.3% (w/v) trypsin (Sigma-Aldrich, St. Louis, MO, USA) was then infused into the uterine lumen through the catheter.

After collection of the epithelial cells, the uterine lumen was washed with sterile HBSS supplemented with antibiotics and 0.1% (w/v) BSA. The horn was then cut transversely with scissors into several segments, which were slit to expose the endometrial surface. Intercaruncular endometrial strips were dissected from the myometrial layer with a scalpel and washed once in 50 mL of sterile HBSS containing antibiotics. The endometrial strips were then minced into small pieces (1 mm^3). The minced tissues (~5 g) were digested by stirring for 60 min in 50 mL of sterile HBSS containing 0.05% (w/v) collagenase, 0.005% (w/v) DNase I (BBI Enzymes, Cardiff, UK) and 0.1% (w/v) BSA. The dissociated cells were filtered through metal meshes (100 µm and 80 µm) to remove undissociated tissue fragments. The filtrate was washed three times by centrifugation (4°C, 10 min at 100g) with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with antibiotics and 0.1% (w/v) BSA. After the washes, the cells were counted using a haemocytometer. The cell viability was greater than 85% as assessed by 0.5% (w/v) Trypan blue dye exclusion.

Cell culture

The final pellets of the epithelial and stromal cells were separately resuspended in culture medium (DMEM/Ham's F-12, 1 : 1 (v/v); Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) bovine serum (Invitrogen), 20 μg mL^{-1} gentamicin (Sigma-Aldrich) and 2 μg mL^{-1} amphotericin B (Sigma-Aldrich) [22]. Epithelial cells were seeded at a density of 1×10^5 viable cells mL^{-1} in culture flasks (Greiner Bio-One, Frickenhausen, Germany) and stromal cells were seeded at a density of 1×10^5 viable cells mL^{-1} in 4-well cluster dishes (Thermo Fisher Scientific, Yokohama, Japan), 24-well cluster dishes (Greiner Bio-One), and 75 cm^2 culture flasks (Greiner Bio-one; #658175) and were cultured at 38.5°C in a humidified atmosphere of 5% CO_2 in air. For the stromal preparation, the medium was changed 2 h after plating, by which time selective attachment of stromal cells had occurred [22, 23]. Alternatively, since the epithelial cells attached 24–48 h after plating, the medium in the epithelial cell culture was replaced 48 h after plating. The medium was changed every 2 days until the cells reached confluence. When the epithelial cells were confluent, 0.02% trypsin solution was added to the cells to collect the pure epithelial cells. Epithelial cells were seeded at a density of 1×10^5 viable cells mL^{-1} in 4-well cluster dishes and 24-well cluster dishes and were cultured at 38.5°C in a humidified atmosphere of 5% CO_2in air until the cells reached confluence. When these cells were confluent (6–7 days after the start of the culture), the medium was replaced with fresh DMEM/Ham's F-12 supplemented with 0.1% (w/v) BSA, 5 ng mL^{-1} sodium selenite (Sigma-Aldrich), 0.5 mM ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan), 5 mg mL^{-1} transferrin (Sigma-Aldrich), 2 mg mL^{-1} insulin (Sigma- Aldrich) and 20 mg mL^{-1} gentamicin.

Culture of endometrial tissues

For tissue culture, endometrial tissues were obtained at the mid luteal stage and at the follicular stage. Endometrial tissue culture was conducted as described previously [24]. Briefly, endometrial strips were washed three times in sterile saline solution containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). The tissues were finally cut into small pieces with scalpel and subsequently washed another three times in Hanks balanced salt solution supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 0.1% BSA. After hanging the tissues with steel needles (TOP, Tokyo, Japan; 8N01B), the individual endometrial tissues with were placed into culture glass tubes (12 mm x 75 mm; Kimble Chase Life Science and Research Products LLC., New Jersey, USA; 73500-13100) containing 2 ml culture medium (DMEM/Ham's F-12; 1:1 (v/v) [Invitrogen; 12400-024]). Supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 0.1% BSA with 5% CO2 in air. Endometrial tissues were exposed to Cr (10-100 nM) at 37.5 C for 4 h. At the end of incubation, 1 ml of the conditioned media were collected into 1.5 ml tubes containing 10 μl of a stabilizer solution (0.3 M EDTA, 1% (W/V) acid acetyl salicylic pH 7.3) and frozen at -30 C. The tissues were blotted on filter paper and weighed to normalize PGF concentration. The concentrations of PGF in the culture media after incubation were determined by enzyme immunoassay (EIA).

Enzyme immunoassay

The concentrations of PGF and PGE2 in the culture medium were determined by an enzyme immunoassay as described previously [25, 26]. The PGF standard curve

ranged from 0.016 to 4 ng mL^{-1} and the median effective dose (ED_{50}) of the assay was 0.25 ng mL^{-1}. The intra- and inter-assay coefficients of variation were, on average, 3.94 and 13.2%, respectively. The PGE2 standard curve ranged from 0.039 to 10 ng mL^{-1} and the ED_{50} of the assay was 0.625 ng mL^{-1}. The intra- and inter-assay coefficients of variation were, on average, 1.36 and 16.7%, respectively.

Reverse transcription and real-time PCR

Total RNA was extracted from cultured cells using TRIsure (Bioline, London, UK) according to the manufacturer's directions. One microgram of each total RNA was reverse transcribed using a ThermoScript RT-PCR System (Invitrogen) and 10% of the reaction mixture was used in each PCR reaction using specific primers for GC-Rα, PGR, PAQR7, PGRMC1, PGRMC2, SERBP1, cytosolic phospholipase A2, group IVA (PLA2G4), COX2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the bovine sequence (Table 1). The primers were chosen using Primer 3, an online software package (http://primer3.sourceforge.net/). Gene expression was measured by real-time PCR using the Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) and the QuantiTect SYBR Green PCR system (Qiagen GmbH, Hilden, Germany) starting with 2 ng of reverse-transcribed total RNA as described previously [27]. GAPDH expression was used as an internal control. For quantification of the mRNA expression levels PCR was performed under the following conditions: 95°C for 15 min followed by 55 cycles of 94°C for 15 s, 55°C for 20 s and 72°C for 15 s. Use of the QuantiTect SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson product moment correlation coefficient, $r > 0.99$).

Western blotting analysis

GC-Rα protein levels in endometrial tissues and stromal cells were assessed by western blotting analysis. Endometrial tissues were homogenized on ice in the homogenization buffer by a tissue homogenizer (Physcotron; Niti-on Inc., Chiba, Japan; NS-50), followed by filtration with a metal wire mesh (150 mm). For GC-Rα protein analysis, nuclei were isolated from the tissue homogenates by centrifugation at 600 x g for 30 min. The cultured stromal cells were lysed in 200 μl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol [Sigma-Aldrich; G7757], Complete, pH 7.4). Protein concentration was determined by the method of (Osnes et al., 1993), using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (10% glycerol, 1% β-mercaptoethanol [Wako Pure Chemical Industries; 137-06862], pH 6.8), and heated at 95 C for 10 min. Samples (30 μg protein) were subjected to SDS-PAGE (12%) for 1.5 h at 200 V. The separated proteins were electrophoretically transblotted to a nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ; USA; RPN78D) for 3 h at 250 mA in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, pH 7.5, 137 mM NaCl]), incubated in blocking buffer (4% nonfat dry milk in TBS-T) overnight at 4 C, incubated at room temperature with a primary antibody specific to each protein (GC-Rα antibody [95 kDa; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; sc-1002; 1:200, 1 h] and ACTB antibody [42 kDa; Sigma-Aldrich; A2228; 1:4000, 1 h]), incubated in blocking buffer

for 10 min at room temperature, washed two times for 10 min in TBS-T at room temperature, incubated with secondary antibody [GC-Rα (1:10 000): anti-rabbit Ig, HRP-linked whole antibody produced in donkey, Amersham Biosciences Co.; NA934; ACTB (1:40 000): anti-mouse Ig, HRP-linked whole antibody produced in sheep, Amersham Biosciences Co.; NA931] for 1 h, and washed two times in TBS-T for 10 min and then washed in TBS for 10 min at room temperature. The signal was detected by ECL Western Blotting Detection System (Amersham Biosciences Co.; RPN2109).

The intensity of the immunological reaction (GC-Rα and ACTB) in the tissues was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, USA).

Statistical analysis

All experimental data are shown as the mean ± s.e.m. of values obtained in three to six separate experiments, where each experimental triplicate was performed using stromal cells from a single bovine endometrium. The statistical significance of differences was assessed by analysis of variance (ANOVA) followed by a Fisher's protected least-significant difference procedure (PLSD) or by a Bonferroni–Dunn test for multiple comparisons using StatView (SAS Institute, Cary, NC, USA).

Table 1. Primers for real-time PCR.

Gene	Primer (5'-3')[a]	Accession no.	Product (bp)
PGR	F: AACGAAAGCCAAGCCCTAAG	AY656812	147
	R: GCTGGAGGTATCAGGTTTGC		
PAQR7	F: CTGGAAGCCGTATATCTACGT	XM005203177.1	286
	R: GCTGTAATGCCAGAACTCGGAC		
PGRMC1	F: TCTTCAGGGGTGTGTGTGAA	NM001075133.1	266
	R: CATTGTCCTGTGCTCTTTGG		
PGRMC2	F: TGCCTCTTTGCCTCGTATGA	NM001099060.1	179
	R: GAGGCATCCCTACCAGCAAAT		
SERBP1	F: AGCTCAGACCAACTCCAATGC	NM001046449.1	149
	R: CGGCTCAGACCTTCTTTCTTCA		
PLA2G4	F: AGGTGCACAACTTCATGCTG	BC134610	107
	R: GGCATCCAATTCGTCTTCAT		
COX2	F: TGTGAAAGGGAGGAAAGAGC	AF004944	115
	R: GGCAAAGAARGCAAACATCA		
GC-Rα	F: CCATTTCTGTTCACGGTGTG	AY238475	132
	R: CTGAACCGACAGGGAATTGGT		
GAPDH	F: CACCCTCAAGATTGTCAGCA	BC102589	103
	R: GGTCATAAGTCCCTCCACGA		

[a] F, forward; R, reverse

CHAPTER 3

GENOMIC AND NON-GENOMIC EFFECTS OF PROGESTERONE ON PROSTAGLANDIN (PG) F2α AND PGE2 PRODUCTION IN THE BOVINE ENDOMETRIUM

INTRODUCTION

Prostaglandin (PG) F2α (PGF) is synthesised and secreted from the endometrium to induce luteolysis and to modulate the oestrous cycle in the cow [5-7]. In contrast, PGE2 has anti-luteolytic activity in the sheep [14]. In the bovine endometrium, epithelial cells principally produce PGF and stromal cells produce PGE2 [28, 29]. The luteolytic pulses of PGF are produced by endometrium in response to oxytocins (OT) binding to OT receptors (OTRs) [30, 31]. Progesterone (P4) acts to block expression of oestrogen receptor and OTR to prevent luteolysis (Spencer and Bazer 2004). However, continuous exposure of the endometrium to P4 downregulates P4 receptor expression in endometrial epithelial cells, which allows the rapid increase of OTR expression [32].

The synthesis of PGs is initiated by arachidonate isolation from plasma membrane phospholipids by phospholipase A2 (PLA2) [8]. Cyclooxygenase-2 (COX2) converts arachidonic acid to PGH2 and then two specific downstream enzymes, PGE synthase (PGES) and PGF synthase (PGFS), catalyse the conversion of PGH2 to PGE2 or PGF, respectively [10]. Thus, the change in expression of these enzymes influences endometrial PG production.

Progesterone is a sex-steroid hormone that is abundantly secreted from the corpus luteum (CL) to establish and maintain pregnancy [5]. Progesterone acts as a direct transcription factor and enhances the transcriptional activity of nuclear P4 receptor (PGR) via the activation of the Ras/Raf/Mek/Erk cascade by binding to the PGR (genomic effect) [33, 34]. On the other hand, there is some evidence that P4 also has rapid effects (non-genomic effects) due to cell membrane-initiated signalling pathways in several cell types [35, 36]. Rapid actions at the membrane level are thought to activate intracellular transduction pathways that induce the synthesis of transcriptional factors required for the genomic effect of P4 [37]. Progesterone binding to progestin/adipoQ receptor family member VII (PAQR7), also known as membrane P4 receptor α, stimulates Erk activity [38, 39]. In addition, P4 receptor membrane component 1 (PGRMC1), when it interacts with serpine 1 mRNA-binding protein (SERBP1), inhibits Erk activity [40], while only minimal information is available for PGRMC2 [41]. The Ras/Raf/Mek/Erk cascade, which is known as the traditional mitogen-activated protein kinase (MAPK) pathway, phosphorylates PG synthases [42].

In the present study, to clarify whether P4 modulates PG synthesis through genomic or non-genomic pathways in bovine endometrial cells, we investigated: (1) the effect of P4 on PGF and PGE2 production by cultured endometrial cells, (2) time-dependent expression of enzymes associated with the arachidonate cascade after P4 treatment in cultured endometrial cells, (3) the effect of bovine serum albumin (BSA)-conjugated P4 (P4-BSA) on PGF and PGE2 production and (4) the expression of PGR, PAQR7, PGRMC1/2 and SERBP1 in bovine endometrium throughout the

oestrous cycle.

MATERIALS AND METHODS

Experiment 1: effect of P4 on endometrial PGF and PGE2 production in cultured bovine endometrial cells

To reveal the effect of P4 on PGF and PGE2 production, endometrial epithelial and stromal cells were exposed to P4 (0, 1, 10 or 100 nM) for 24 h. PGF and PGE2 concentrations in the media of both cells were measured by enzyme immunoassay. DNA content was measured by the spectrophotometric method [43] and used to standardise the results.

Experiment 2: effect of P4 on PLA2 and COX2 mRNA expression in cultured bovine endometrial cells

To elucidate whether P4 affects *PLA2* and *COX2* mRNA expression in bovine endometrial epithelial and stromal cells, both cell types were cultured with P4 (0, 1, 10 or 100 nM) for 24 h. After incubation, total RNA was extracted for determination, then *PLA2* and *COX2* mRNA expression was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR).

Experiment 3: short-term effect of P4 on PLA2 and COX2 mRNA expression in cultured bovine endometrial cells

To clarify whether *PLA2* and *COX2* mRNA expression was influenced by P4 in a very short time, endometrial epithelial and stromal cells were pre-incubated with fresh medium for 1 h. After incubation, the medium was replaced with fresh medium and cells were exposed to P4 (0 or 10 nM) for 5, 10, 15 or 20 min. Total RNA was extracted for determination, then *PLA2* and *COX2* mRNA expression was examined by quantitative RT-PCR.

Experiment 4: non-genomic effect of P4 on endometrial PGF and PGE2 production in cultured bovine endometrial cells

To reveal the effect of membrane-mediated P4 on PGF and PGE2 production, endometrial cells were cultured with 0, 1, 10 or 100 nM P4-BSA (Sigma-Aldrich) for 24 h. P4-BSA specifically binds to cell surface membranes. PGF and PGE2 concentrations in the media of both cell types were measured by enzyme immunoassay. DNA content was measured by the spectrophotometric method [43] and used to standardise the results.

Experiment 5: PGR, PAQR7, PGRMC1, PGRMC2 and SERBP1 mRNA expression in the bovine endometrium throughout the oestrous cycle

Expression of *PGR*, *PAQR7*, *PGRMC1*, *PGRMC2* and *SERBP1* mRNA in endometrial tissues of each stage (n = 5 per stage) were examined by quantitative RT-PCR.

RESULTS

Experiment 1: Effect of P4 on endometrial PGF and PGE2 production in cultured bovine endometrial cells

Progesterone (100 nM) increased only PGE2 production compared to controls in cultured bovine endometrial epithelial cells (Figure 1C: $P<0.05$). Gradual decreases of PGF and PGE2 production were observed after P4 treatment in the stromal cells (Figure 1B, D: $P<0.05$). The ratio of PGF to PGE2 production was suppressed by P4 (10, 100 nM) in the stromal cells (Figure 1F: $P<0.05$) but there was no difference in the epithelial cells (Figure 1E).

Experiment 2: Effect of P4 on PLA2 and COX2 mRNA expression in cultured bovine endometrial cells

Progesterone (1-100 nM) decreased both *PLA2* and *COX2* mRNA expression dose-dependently in cultured bovine endometrial stromal cells (Figure 2B, D: $P<0.05$) but not in epithelial cells (Figure 2A, C).

Experiment 3: Short-term effect of P4 on PLA2 and COX2 mRNA expression in cultured bovine endometrial cells

There was no effect on *PLA2* and *COX2* mRNA expression by exposing P4 (10 nM) for a short-term (5, 10, 15, 20 min) in both cultured endometrial cells (Figure 3).

Experiment 4: Non-genomic effect of P4 on endometrial PGF and PGE2 production in cultured bovine endometrial cells

Progesterone-BSA (100 nM) significantly increased PGF and PGE2 production in cultured bovine endometrial epithelial cells (Figure 4A, C: $P<0.05$) but not in stromal cells (Figure 4B, D). There was no difference the ratio of PGF to PGE2 production in bovine endometrial epithelial and stromal cells (Figure 4E, F).

Experiment 5: PGR, PAQR7, PGRMC1, PGRMC2, and SERBP1 mRNA expression in the bovine endometrium throughout the estrous cycle

PGR, *PAQR7*, *PGRMC1*, *PGRMC2*, and *SERBP1* mRNA expressions were detected in the bovine endometrium throughout the estrous cycle (n=5 cows/ stage). *PGR* mRNA expression was higher at the follicular phase (Days 19-21) than at the early (Days 2-3) to mid (Days 8-12) luteal stages (Figure 5A). There were no changes in *PAQR7*, *PGRMC1*, *PGRMC2*, and *SERBP1* mRNA expression during the estrous cycle (Figure 5B-E).

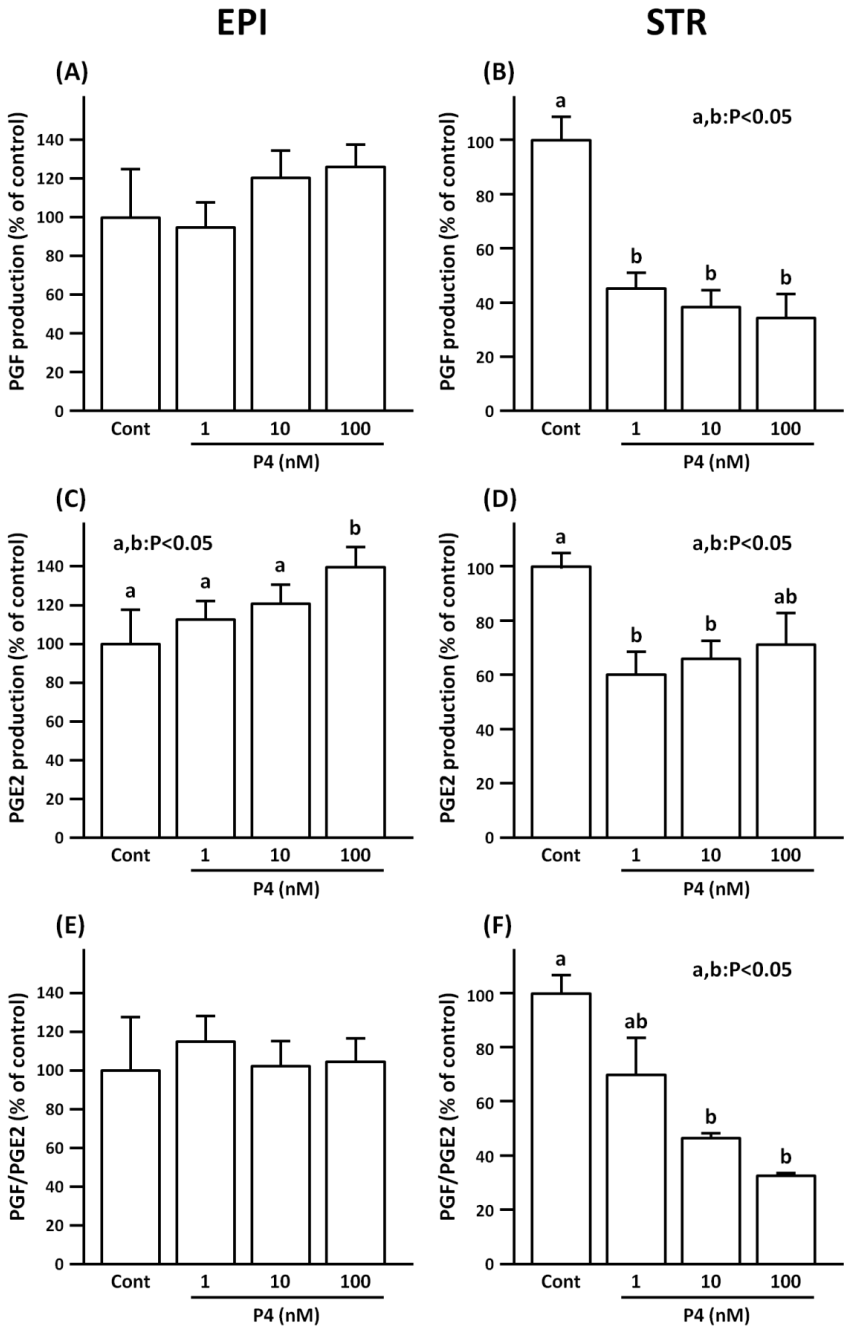

Figure 1. Mean (± s.e.m.) secretion of PGF, PGE2 and the ratio of PGF to PGE2 by endometrial epithelial and stromal cells on Days 2–5 of the bovine oestrous cycle (n = 3–8). Cultured bovine endometrial (*a*, *c*, *e*) epithelial and (*b*, *d*, *f*) stromal cells were incubated for 24 h with P4. Different superscript letters indicate significant difference ($P < 0.05$) as determined by ANOVA followed by a Bonferroni–Dunn as a multiple-comparison test.

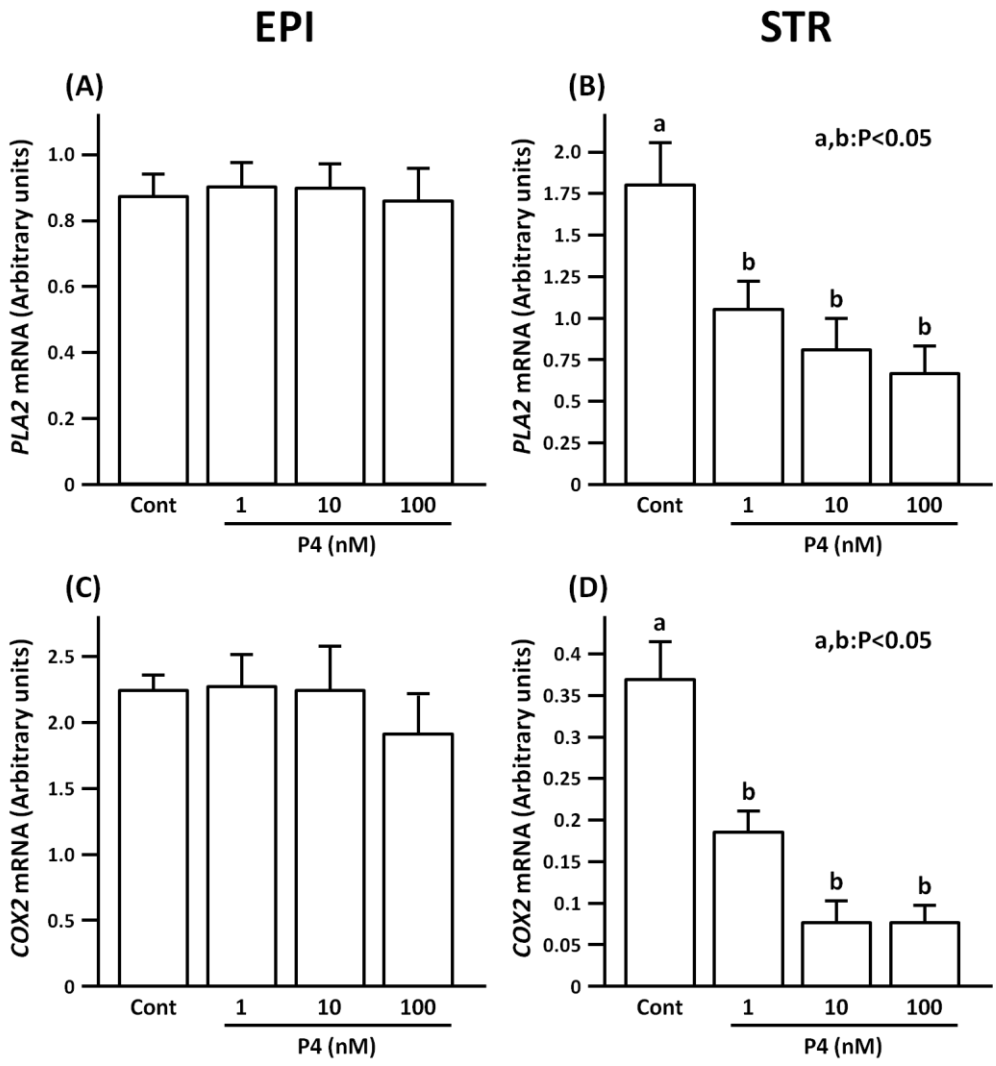

Figure 2. Effects of progesterone (P4) on the level of *PLA2* and *COX2* mRNA expression in cultured bovine (*a*, *c*) epithelial and (*b*, *d*) stromal cells. Endometrial cells were exposed to P4 (1–100 nM) for 24 h. All values (mean ± s.e.m.) are expressed as the relative ratio of *PLA2* and *COX2* mRNA to *GAPDH* mRNA. Different letters indicate significant difference ($P < 0.05$) as determined by ANOVA followed by a Bonferroni–Dunn as a multiple-comparison test.

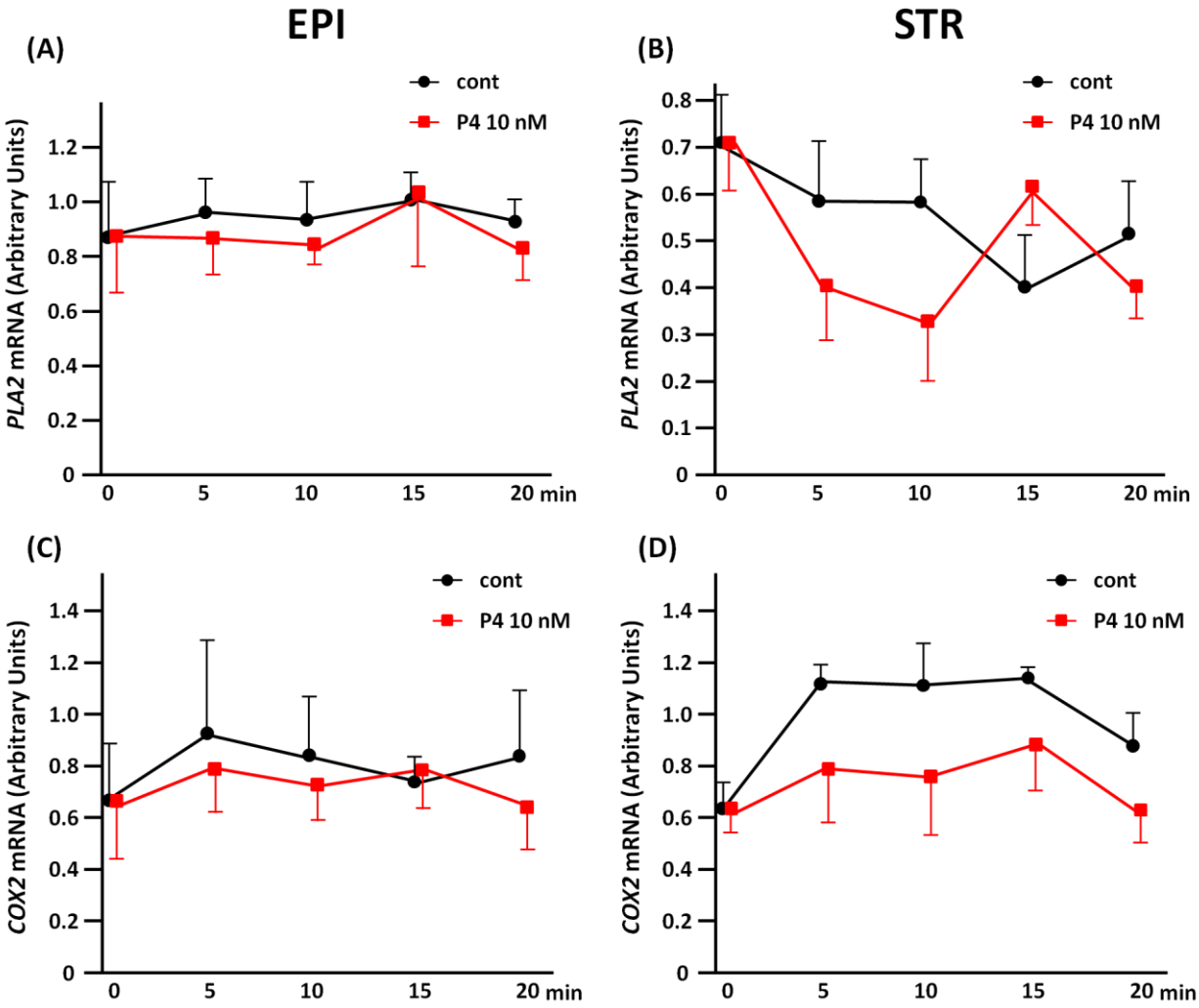

Figure 3. Time-dependent effects of progesterone (P4) on *PLA2* and *COX2* mRNA expression in cultured bovine endometrial (*a*, *c*) epithelial and (*b*, *d*) stromal cells. After 1 h of pre-incubation, both endometrial cell types were exposed to 10 nM P4 for 0, 5, 10, 15 or 20 min. All values (mean ± s.e.m.) are expressed as the relative ratio of *PLA2* and *COX2* mRNA to *GAPDH* mRNA. Different letters indicate significant difference ($P < 0.05$) as determined by ANOVA followed by a Bonferroni–Dunn as a multiple-comparison test.

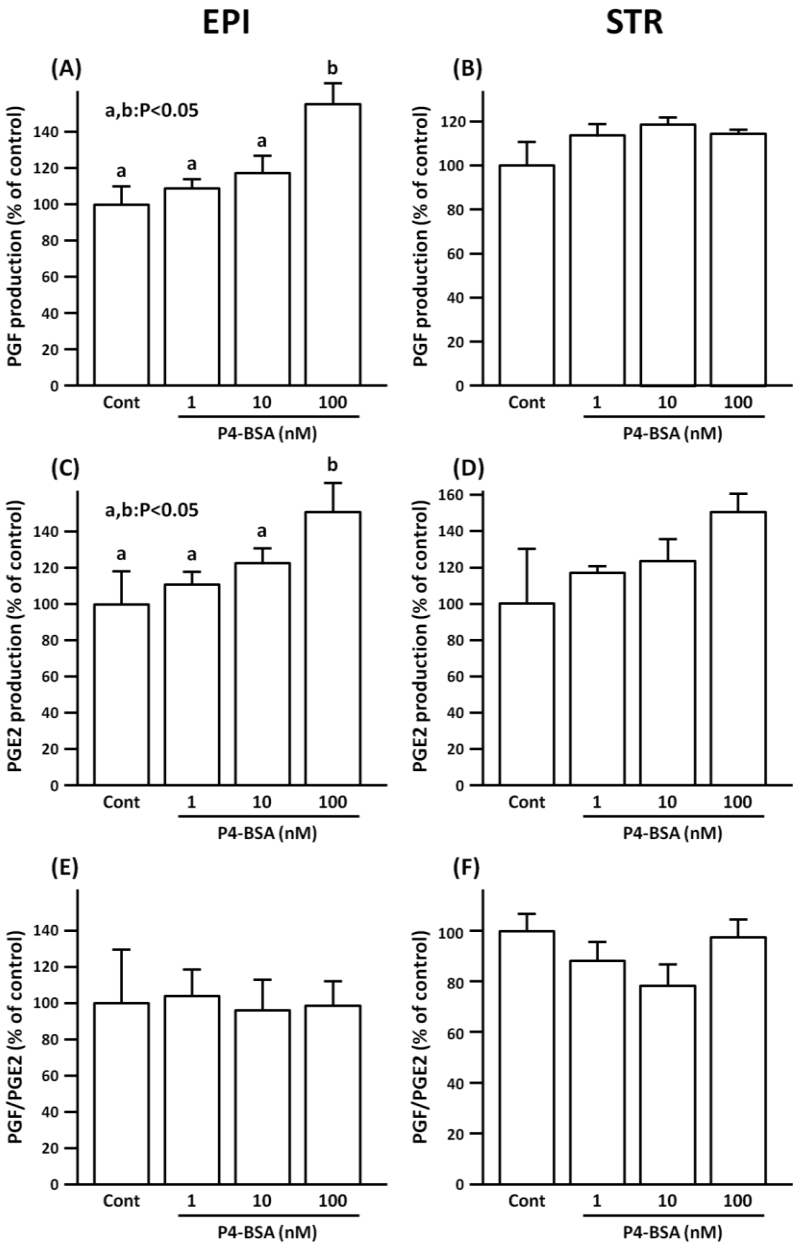

Figure 4. Mean (± s.e.m.) secretion of PGF, PGE2 and the ratio of PGF to PGE2 by endometrial epithelial and stromal cells on Days 2–5 of the bovine oestrous cycle (n = 3–8). Cultured bovine endometrial (*a*, *c*, *e*) epithelial and (*b*, *d*, *f*) stromal cells were incubated for 24 h with P4-BSA. Different superscript letters indicate significant difference ($P < 0.05$) as determined by ANOVA followed by a Bonferroni–Dunn as a multiple-comparison test.

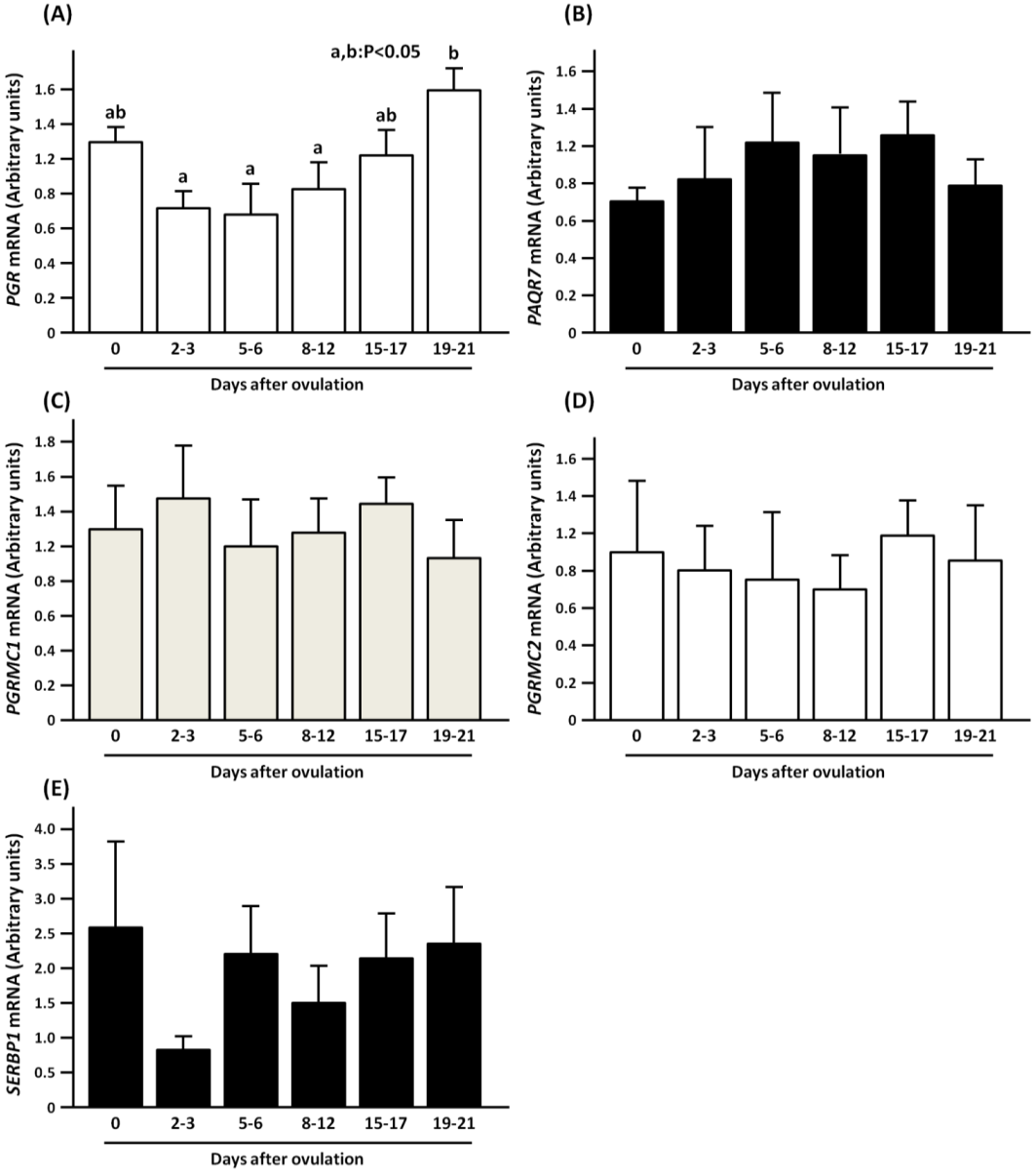

Figure 5. Expression of (*a*) *PGR*, (*b*) *PAQR7*, (*c*) *PGRMC1*, (*d*) *PGRMC2* and (*e*) *SERBP1* mRNA in bovine endometrium throughout the oestrous cycle (oestrus, Day 0; early-luteal, Days 2–3; developing, Days 5–6; mid-luteal, Days 8–12; late-luteal, Days 15–17; follicular stage, Days 19–21). All values (mean ± s.e.m.) are expressed as the relative ratio of receptor expression to *GAPDH*. Different superscript letters indicate significant difference ($P < 0.05$) as determined by ANOVA followed by a Bonferroni–Dunn as a multiple-comparison test.

DISCUSSION

In previous studies, P4 treatment for a long time (72 h) stimulated both PGF and PGE2 production in cultured bovine endometrial epithelial cells, but did not affect stromal cells [29, 44]. Furthermore, P4 inhibited OT-stimulated PGF secretion both from endometrial slices and from epithelial cells during 4 h of incubation [45]. Moreover, P4 pre-treatment for 30 min reduced intracellular mobilisation of Ca^{2+} in response to OT [45]. This effect was supposed to occur via activation of a membrane receptor that belongs to the class of membrane receptors coupled to phospholipase C. In the present study, P4 treatment (24 h) significantly stimulated PGE2 production but not PGF production by cultured endometrial epithelial cells, whereas P4 significantly inhibited PGF and PGE2 production and the ratio of PGF to PGE2 production by stromal cells. The findings suggest that the endometrial cellular reaction to P4 changes according to exposure time. Thus, it is important to clarify the extensive mechanisms of intracellular P4 function. Prostaglandin biosynthesis begins with PLA2, which leads to the release of arachidonic acid from plasma membrane phospholipids [46], and then COX2 converts free arachidonic acid to PGH2 [47]. Therefore, both enzymes act as important physiological control points of PG production. In the present study, P4 inhibited *PLA2* and *COX2* mRNA expression in cultured endometrial stromal cells but not in epithelial cells. The suppression of PGF and PGE2 production by P4 in Experiment 2 seems to be due to the downregulation of *PLA2* and *COX2* mRNA expression in bovine endometrial stromal cells. In the epithelial cells, P4 did not affect the expression of these enzymes, although P4 stimulated PGE2 production. These findings suggest that P4 stimulates the expression and activation of other factors involved in PGE2 production in bovine endometrial epithelial cells. In stromal cells, short-term (5, 10, 15 or 20 min) P4 treatment did not affect either *PLA2* or *COX2* mRNA expression, although P4 treatment for 24 h suppressed *PLA2* and *COX2* mRNA expression in Experiment 3. On the other hand, short-term and 24-h exposure to P4 did not affect either *PLA2* or *COX2* mRNA expression in epithelial cells. These findings suggest that the effect of P4 on the production of PGs is induced via a PGR-activating pathway in bovine endometrial stromal cells.

A membrane-initiated action of P4 was demonstrated using P4-BSA, because P4 covalently linked to BSA cannot enter the cells and its binding is restricted to cell-surface membrane progesterone receptors [40, 48]. In the present study, P4-BSA stimulated PGF and PGE2 production in epithelial cells but not in stromal cells. The above findings suggest that P4 dominantly binds membrane receptors to stimulate production of PGs in epithelial cells, whereas P4 selectively binds to PGR in stromal cells to suppress production of PGs.

In the present study, PGR expression was higher at the follicular phase than at the early- to mid-luteal stages. The expression patterns of P4 receptors, except SERBP1, throughout the oestrous cycle are consistent with those observed in a previous study [49]. Following ovulation, P4 concentrations begin to increase due to the formation of the CL. The granulosa and theca cells of the ovulated dominant follicle luteinise and produce P4 in readiness for the establishment and maintenance of pregnancy or resumption of the oestrous cycle [50]. Endometrial P4 concentration is highest on Days 1–5 of the oestrous cycle and it subsequently decreases and is maintained at a low level during the remainder

of the cycle [49]. These findings suggest that the genomic effect of P4 on endometrial PGF production may be mediated by interaction with PGR to protect the CL against endometrial PGF production. Rapid effects of P4 through the membrane P4 receptor is thought to continuously induce expression of various genes throughout oestrous cycle.

SUMMARY

Progesterone (P4) acts through different actuating pathways called genomic and non-genomic pathways. Here we investigated whether P4 regulates prostaglandin (PG) F2α (PGF) and PGE2 production in bovine endometrium through different pathways. Cultured endometrial cells were exposed to P4 for a short time (5–20 min) or bovine serum albumin (BSA)-conjugated P4 (P4-BSA) for 24 h. Progesterone treatment for 24 h stimulated PGE2 production in epithelial cells, but suppressed both PGF and PGE2 production and the expression of PG-metabolising enzymes including phospholipase A2 (PLA2) and cyclooxygenase-2 (COX2) in stromal cells. Short-term (5–20 min) P4 treatment did not affect *PLA2* or *COX2* transcript levels in either cell type. P4-BSA increased PGF and PGE2 production only in epithelial cells. *Nuclear P4 receptor* mRNA expression in endometrium was higher at the follicular phase than at the early- to mid-luteal stages, whereas *membrane P4 receptor* mRNA expression did not change throughout the oestrous cycle. The overall results suggest that P4 controls PG production by inhibiting enzymes via a genomic pathway and by stimulating signal transduction via a non-genomic pathway. Consequently, P4 may protect the corpus luteum by attenuating PGF production in stromal cells and by increasing PGE2 secretion from epithelial cells.

CHAPTER 4

EXPRESSION OF GLUCOCORTICOID RECEPTOR α AND ITS REGULATION IN THE BOVINE ENDOMETRIUM: POSSIBLE ROLE IN CYCLIC PROSTAGLANDIN F2α PRODUCTION

INTRODUCTION

The bovine endometrium synthesizes and secretes prostaglandin F2α (PGF), which is the main luteolysin responsible for regression of corpus luteum (CL). In non-pregnant cows, the uterus increases PGF production between Days 17 and 19 post-ovulation [4]. The increase in uterine PGF production and its pulsatile release seem to regulate the estrous cycle by inducing luteal regression in ruminants [5-7]. We reported that the amount of PGF production by the bovine endometrium was low at the mid luteal stage, increased at the late luteal stage and reached the highest levels at estrus [20]. Thus, PGF production and secretion by the endometrium seem to be precisely regulated throughout the estrous cycle.

Glucocorticoids (GCs) derived from the adrenal cortex have been shown to regulate female reproductive functions [51, 52]. Recently, we suggested that active GC, cortisol (Cr), affects pregnancy rate and CL function in cattle [53]. In addition, Cr suppresses basal and PGF production stimulated by tumor necrosis factor-α in the bovine endometrial stromal cells but not in the epithelial cells [24]. Moreover, 11β-hydroxysteroid dehydrogenases (HSD11B) play an important role in regulating GC availability in target tissues. HSD11B type 1 mainly converts cortisone to Cr in the target organs [54, 55]. A previous study demonstrated that the activity of HSD11B in the bovine endometrium was greater at the follicular stage and estrus than at the other stages of the estrous cycle [24]. In addition, PGF increased the protein expression of HSD11B in bovine endometrial stromal cells [24]. The above findings suggest that Cr plays some important roles in regulating PGF production throughout the estrous cycle in the bovine endometrium.

The above findings raise the possibility that the intra-cellular availability of Cr increases, and thereby PGF production is suppressed, when HSD11B expression and activity are high at the follicular stage. However, PGF production by the bovine endometrium is highest at the follicular stage [20, 24]. These results suggest that the inhibitory effect of Cr on PGF production depends on other factors. The biological action of Cr is mediated through the activation of specific intracellular receptors, GC receptors (GC-R) [56-60]. Because Cr actions depend on the expression of GC-Rα in the target organs, endometrial PGF production appears to be regulated by not only changes in the levels of active GC (Cr) but also in GC-Rα expression throughout the estrous cycle. However, the regulatory mechanisms controlling the cyclic changes of endometrial PGF production remain unclear.

Progesterone (P4) and estradiol-17β (E2) secreted by the ovary play crucial rolles in regulating functional and structural changes in the endometrium throughout the estrous cycle [61]. Furthermore, in ruminants, the uterine epithelium, stroma and myometrium all contain receptors for P4 and E2 [62]. Thus, we hypothesize that P4 and E2 regulate GC-Rα expression which plays an important role in controlling PGF

production in the bovine endometrium.

In the present study, to determine the regulatory mechanisms of PGF production in the bovine endometrium, we investigated 1) GC-Rα protein expression and the biological actions of Cr in bovine endometrium throughout the estrous cycle and 2) the roles of steroid hormones in the regulation of GC-Rα expression in bovine endometrial stromal cells.

MATERIALS AND METHODS

Experiment 6: GC-Rα protein expression throughout the estrous cycle

Expression of GC-Rα protein in endometrial tissues of each stage (n = 3 per stage) were examined by western blotting analysis.

Experiment 7: Effect of Cr on endometrial PGF production at the mid luteal stage and at the follicular stage

To clarify whether biological action of Cr on PGF production is dependent on cyclic changes of GC-Rα, endometrial tissues at the mid luteal stage and at the follicular stage were exposed to Cr (0, 10 nM) for 4 h. PGF concentrations in the media were measured by enzyme immunoassay. The cultured tissues were weighted to normalize PGF concentration.

Experiment 8: Effect of Cr, P4 and E2 on GC-Rα mRNA and protein expression in cultured bovine endometrial stromal cells

To elucidate whether steroid hormones affect GC-Rα mRNA and protein expression in bovine endometrial stromal cells, cells were cultured with Cr (0, 1, 10 or 100 nM) or P4 (0, 0.1, 1, 10 nM) or E2 (0, 0.1, 1, 10 nM) for 24 h. After incubation, the *GC-Rα* mRNA expression was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR). The GC-Rα protein expression was examined by western blotting analysis.

RESULTS

Experiment 6: GC-Rα protein expression throughout the estrous cycle

GC-Rα protein was detected in the bovine endometrium throughout the estrous cycle (n=3 cows/ stage). GC-Rα protein expression was higher at the mid and late luteal stages than at other stages (Figure 6: $P<0.05$).

Experiment 7: Effect of Cr on endometrial PGF production at the mid luteal stage and at the follicular stage

Cr (10 nM) decreased PGF production in endometrial tissue collected at the mid luteal stage ($P<0.05$), but not in endometrial tissue collected at the follicular stage ($P>0.05$) (Figure 7).

Experiment 8: Effect of Cr, P4 and E2 on GC-Rα mRNA and protein expression in cultured bovine endometrial stromal cells

Cr decreased GC-Rα mRNA and protein expression. The effect was significant

at 100 nM in cultured bovine endometrial stromal cells (Figure 8: $P<0.05$). P4 increased GC-Rα mRNA and protein expression. The effect was significant at 10 nM in cultured bovine endometrial stromal cells (Figure 9: $P<0.05$). Exposing cultured bovine endometrial stromal cells to E2 did not affect *GC-Rα* mRNA expression (Figure 10A) but significantly decreased GC-Rα protein expressions (Figure 10B: $P<0.05$).

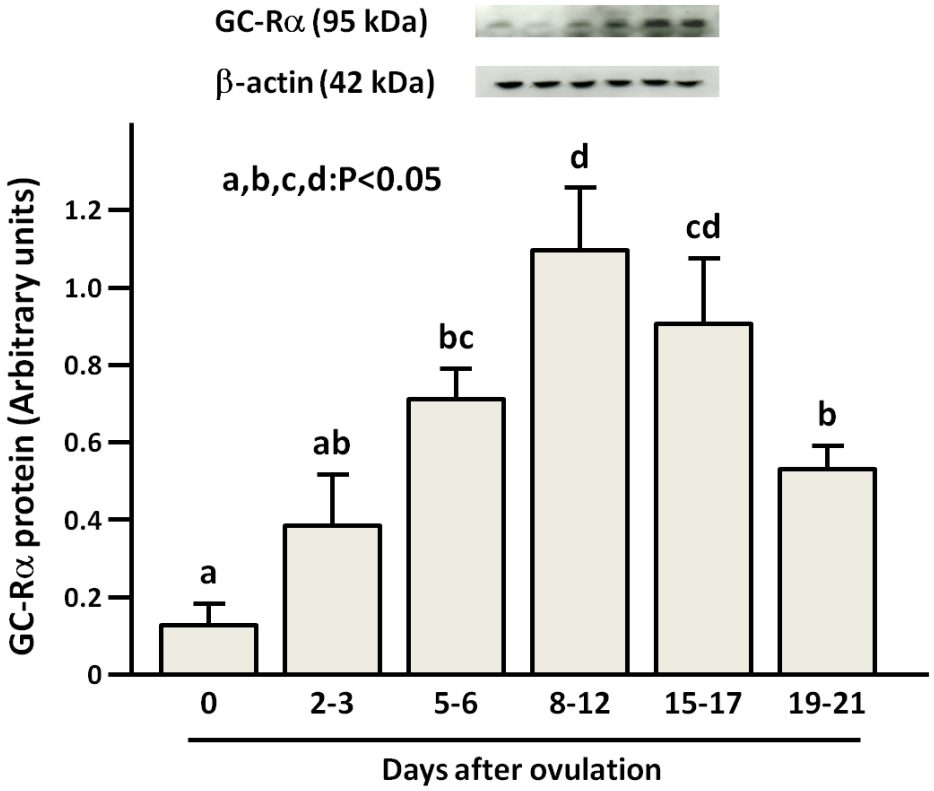

Figure 6. Expression of GC-Rα protein in bovine endometrium throughout the estrous cycle (Estrus, Day 0; early, Days 2-3; developing [Dev], Days 5-6; mid, Days 8-12; late, Days 15-17; follicular stage, Days 19-21). Data are the mean±SEM, for three samples/stage and are expressed as the relative ratio of GC-Rα and ACTB. Representative samples of western blot for GC-Rα and ACTB are shown in the upper panels. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

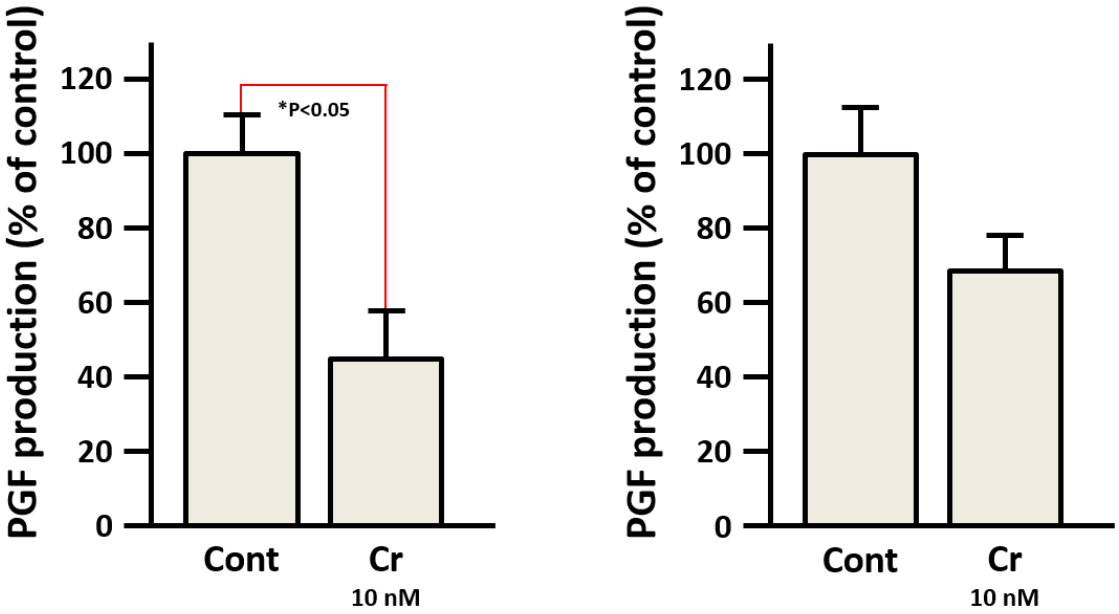

Figure 7. Effect of Cr on the production of PGF in cultured bovine endometrium (A) at the mid luteal stage and (B) at the follicular stage. Endometrial tissues were pre-incubated for 1 h in culture medium and then exposed to cortisol (10 nM) for 4 h. Different superscript letters indicate significant differences ($P<0.05$), as determined by ANOVA followed by a Fisher protected least significant difference (PLSD) procedure as a multiple comparison test.

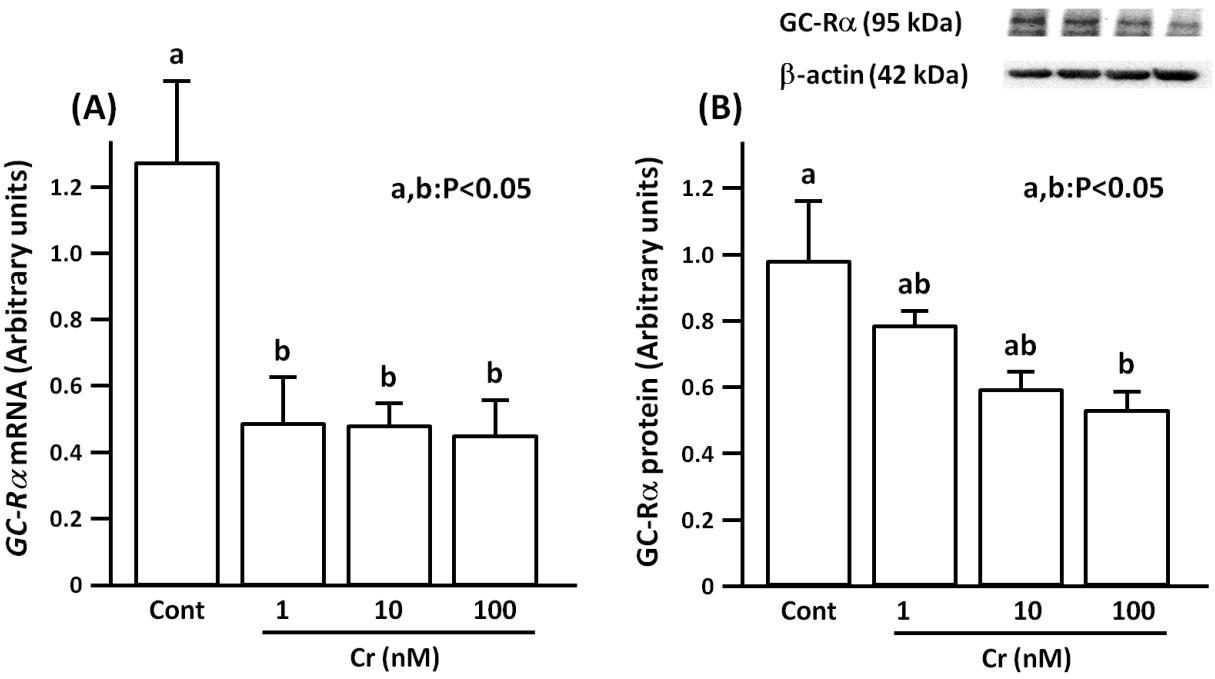

Figure 8. Effect of cortisol on (A) the amounts of *GC-Rα* mRNA and (B) GC-Rα protein expressions in cultured bovine endometrial stromal cells. Endometrial stromal cells were exposed to cortisol (1 - 100 nM) for 24 h. Data are the mean±SEM of four separate experiments performed in triplicate and are expressed as the relative ratio of *GC-Rα* mRNA to *GAPDH* mRNA (A) and GC-Rα to ACTB protein (B). Representative samples of western blot for GC-Rα and ACTB are shown in the upper panels of Fig. 3B. Different letters indicate significant difference (P<0.05), as determined by ANOVA followed by a fisher's PLSD as a multiple comparison test.

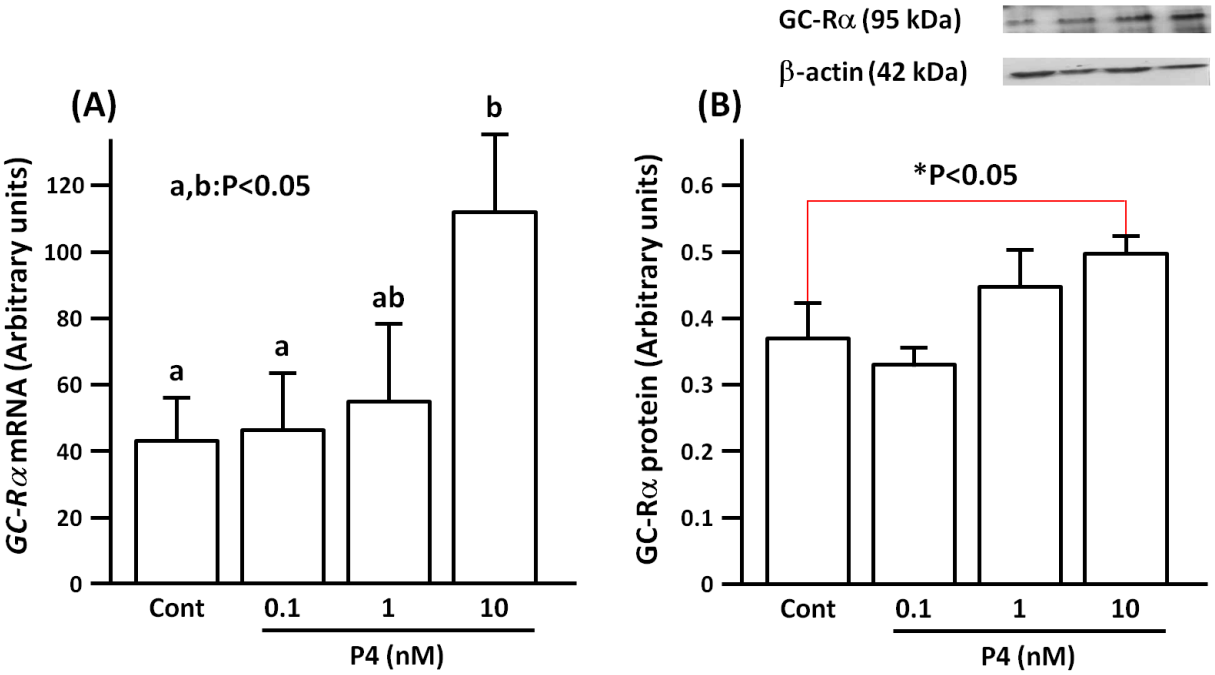

Figure 9. Effect of progesterone on (A) the amounts of *GC-Rα* mRNA and (B) GC-Rα protein expressions in cultured bovine endometrial stromal cells. Endometrial stromal cells were exposed to progesterone (0.1 - 10 nM) for 24 h. Data are the mean±SEM of four separate experiments performed in triplicate and are expressed as the relative ratio of *GC-Rα* mRNA to *GAPDH* mRNA (A) and GC-Rα to ACTB protein (B). Representative samples of western blot for GC-Rα and ACTB are shown in the upper panels of Fig. 4B. Differentletters indicate significant difference (P<0.05), as determined by ANOVA followed by a fisher's PLSD as a multiple comparison test.

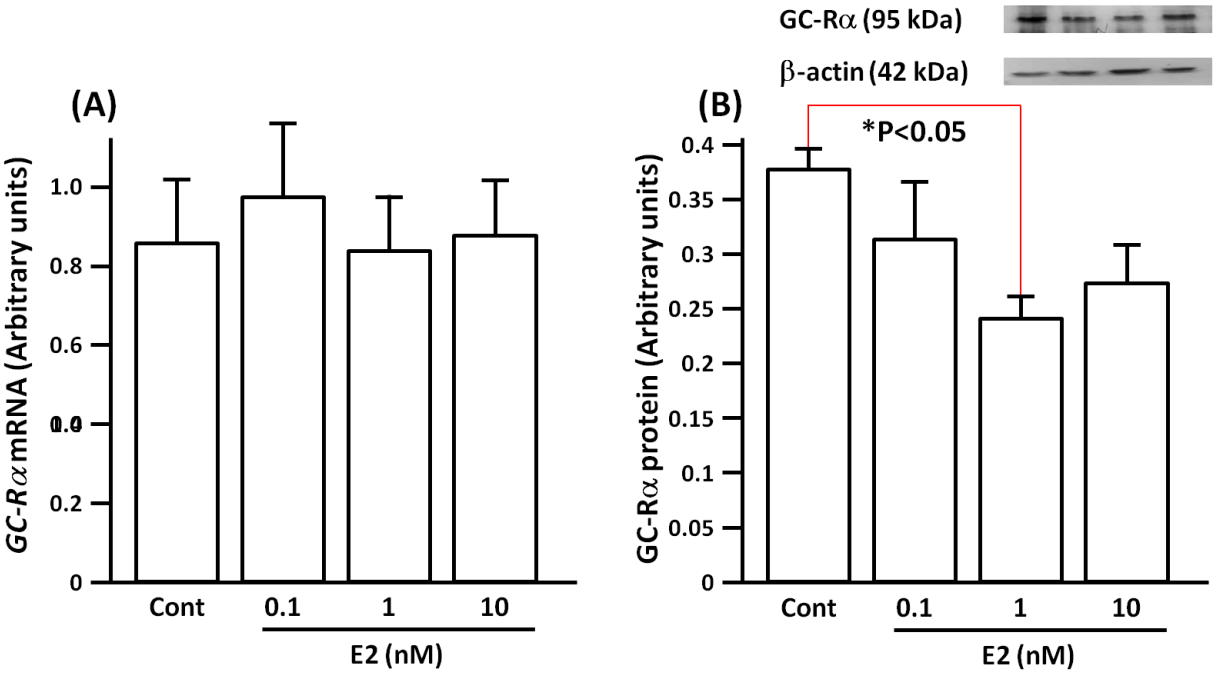

Figure 10. Effect of estradiol-17β on GC-Rα mRNA (A) and protein (B) expression in cultured bovine stromal cells (mean±SEM, n=3 experiments performed in triplicate). Estradiol-17β (0.1-10 nM) were added 24 h before the end of culture. Data are the mean±SEM of four experiments performed in triplicate and are expressed as the ratio of GC-Rα to GAPDH mRNA (A) and GC-Rα to ACTB protein (B). Representative samples of western blot for GC-Rα and ACTB are shown in the upper panels of Fig. 5B. Different superscript letters indicate significant difference (P<0.05), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

DISCUSSION

The biological action of Cr is mediated through intracellular GC-R. Two isoforms of GC-R (GC-Rα and GC-Rβ) originate from the same gene by alternative splicing of the GC-R primary transcript [65-67]. Since the ligand-dependent GC-Rα stimulates gene transcription in Cr target tissues, GC-Rα is thought to be the active receptor isoform [65]. GC-Rα protein was detected in the nuclei of most cell types in the endometrium of cyclic and pregnant cows [68]. In the present study, we examined whether changes of GC-Rα expression during the estrous cycle are associated with PGF production in the bovine endometrium. The protein expression of GC-Rα was significantly higher at the mid luteal stage than at the other stages. Since PGF production is significantly lower at the mid luteal stage than at the follicular stage [20], the cyclic changes of GC-Rα seem to be associated with the regulation of PGF production in the bovine endometrium. In fact, the suppressive effect of Cr on PGF production by the bovine endometrial tissue was greater at the mid luteal stage than at the follicular stage in the present study. These findings strongly suggest that changes in GC-Rα expression are relevant for the suppressive effect of Cr on PGF production in the bovine endometrium.

Glucocorticoids and other steroid hormones down-regulate the levels of their cognate receptors in a number of target tissues and in many different cell lines [69, 70]. GC-Rα was down-regulated by its own ligand in different cell types [71, 72]. This effect is thought to be a feedback protector mechanism that would avoid deleterious effects of prolonged exposure to hormone [73, 74]. In the present study, Cr also inhibited GC-Rα mRNA and protein level in bovine endometrial stromal cells. The above findings suggest that Cr has a role in regulating of GC-Rα expression in bovine endometrial stromal cells. Although the plasma concentration of Cr does not change throughout the estrous cycle [75], the local concentration of Cr in bovine endometrium has been suggested to be regulated by the levels of HSD11B [24]. HSD11B protein expression and activity were greater at the follicular stage than at the mid luteal stage [24]. Thus, the reason why GC-Rα expression was low at estrus and at the follicular stage in the present study, may be due to the downregulation by high level of local Cr.

Both P4 and E2 are sex steroid hormones which regulate several female reproductive functions [76]. GC-Rα mRNA [24] and protein expression observed in our study were highest at the mid luteal stage in the bovine endometrium. The changes of GC-Rα expression throughout the estrous cycle were similar to the cyclic changes of the plasma P4 concentration [5], suggesting that P4 is one of the regulators of GC-Rα expression throughout the estrous cycle. In fact, P4 stimulated GC-Rα expression in cultured stromal cells in the present study. In contrast to P4, plasma E2 concentration increases during the follicular stage and reaches a peak at estrus, when endometrial PGF production is higher than at the other stages of the estrous cycle [22]. E2 has been shown to down-regulate GC-Rα expression and influence the sensitivity to Cr in human breast cancer cells [77]. In agreement with these reports, E2 inhibited GC-Rα protein expression in cultured bovine endometrial stromal cells in the present study, suggesting that E2 regulates GC-Rα expression and consequently interacts with Cr in bovine endometrial stromal cells as it does in other cells. Thus, P4 and E2 may regulate PGF

production via promoting or suppressing the expression of GC-Rα, at least in the stromal cells.

In conclusion, the findings of this study suggest that the expression of GC-Rα is important to regulate PGF production in the bovine endometrium and that sex steroid hormones and Cr control the cyclic changes in endometrial PGF production in bovine endometrium at least in part by regulating GC-Rα expression.

SUMMARY

Cortisol (Cr), the most important glucocorticoid (GC), is well known to suppress uterine prostaglandin F2α (PGF) production. However, the details of the regulatory mechanisms controlling the cyclic changes in endometrial PGF production remain unclear. Here we investigated the expression of GC receptor (GC-Rα), the actions of cortisol throughout the estrous cycle and the regulatory mechanism of GC-Rα in bovine endometrium. The levels of GC-Rα protein were greater at the mid-luteal stage (Days 8-12) than at the other stages. Cr more strongly suppressed PGF production at the mid luteal stage than at the follicular stage. GC-Rα expression was increased by progesterone (P4) but decreased by estradiol-17β (E2) in cultured endometrial stromal cells. The overall results suggest that ovarian steroid hormones control the cyclic changes in endometrial PGF production by regulating GC-Rα expression in bovine endometrial stromal cells.

CONCLUSION

The present study investigated that the regulatory mechanisms of bovine endometrial PGs production. Endometrial PGs are the most important modulator of the estrous cycle and are regulated by steroid hormones. The first series of experiments showed that P4 has some actuating pathways and directly controls PGs production by regulating enzymes of the arachidonate cascade via genomic pathways and by controlling signal transduction via non-genomic pathways. The second series of experiments demonstrated that Cr coordinate with GC-Rα attenuates endometrial PGF production. Moreover, the suppressive effect of Cr on PGF is accelerated by P4 at the mid-luteal stage to prevent luteolysis.

Overall results suggest that endometrial PGs production is regulated by steroid hormones throughout the estrous cycle

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ABSTRACT IN JAPANESE
ウシ子宮内膜における prostaglandin 合成に及ぼす ステロイドホルモンの作用動態に関する研究

久世　真理子

ウシを含む多くの哺乳動物において排卵後の卵巣に形成される黄体は妊娠の成立と維持に必須の progesterone (P4) を分泌する一過性の内分泌器官である。黄体が機能している限り、P4 が上部中枢の働きを抑制することによって次の排卵周期は起こらない。従って、妊娠が不成立の場合、次の妊娠の機会を得るために黄体は速やかに消失 (退行) しなければならない。Prostaglandins (PGs) は炎症反応や筋収縮作用を持つ生理活性物質として知られているが、非妊娠牛において、排卵後 17-19 日目の子宮内膜からパルス状に分泌される prostaglandin F2α (PGF) は強力な黄体退行因子として知られている。一方、胚の存在下において estradiol-17β (E2) の刺激を受けて子宮内膜から分泌される黄体保護因子の prostaglandin E2 (PGE2) は PGF の黄体退行作用を阻害することで妊娠を維持する。様々な動物種においてステロイドホルモンが PGs の重要な調節因子であると考えられている。黄体由来のステロイドホルモンである P4 は子宮内膜における oxytocin (OT) receptor 発現を抑制することで OT による PGF 産生を抑制し、中期黄体の維持に作用することが報告されている。さらに副腎皮質由来のステロイドホルモンである glucocorticoid (GC) は酵素により活性型 GC である cortisol へと転換され、子宮内膜において間質細胞特異的に PGF 産生を抑制することが示されている。しかし、これらのステロイドホルモンの PGs 産生に及ぼす詳細な作用メカニズムは明らかにされていない。

本研究では、ウシ子宮内膜における PGs 合成調節機構を解明する研究の一環として、(1) ウシ子宮内膜上皮および間質細胞における PGs 合成に及ぼす P4 の影響ならびにその作用経路、 (2) ウシ子宮内膜における排卵周期を通じた cortisol の PGF 抑制作用を調べた。本研究で用いた測定系は、PGs 濃度 (enzyme immunoassay: EIA)、遺伝子発現量 (real-time PCR 法)、タンパク質発現量 (western blot 法) である。

(1)ウシ子宮内膜における PGs 合成に及ぼす P4 の核内受容体を介した作用を調べる目的で、ウシ子宮内膜から単離した子宮内膜上皮ならびに間質細胞をコンフルエントになるまで培養した後、P4 を 1, 10, 100 nM 添加し 24 時間後の培養上清中 PGF および PGE2 濃度ならびに PGs 合成酵素 (*phopholipase A2: PLA2* および *cyclooxygenase-2: COX2*) mRNA 発現量を測定した。同細胞に膜受容体特異的に結合する BSA-conjugated P4 (P4-BSA) を 1, 10, 100 nM 添加し 24 時間後の培養上清中の PGF および PGE2 濃度を測定した。排卵周期を通じた P4 受容体発現を調べる目的で、子宮内膜組織を卵巣の肉眼的所見から 6 周期に分類し (排卵日: day 0、黄体初期: days 2-3、黄体形成期: days 5-6、黄体中期:

days 8-12、黄体後期: days 15-17、卵胞期: days 19-21)、各周期における P4 の核内受容体 (*PGR*) および膜受容体 (*PAQR7, PGRMC1, PGRMC2, SERBP1*) mRNA 発現量を測定した。

ウシ子宮内膜間質細胞において P4 は PGF および PGE2 合成ならびに *PLA2* および *COX2* mRNA 発現を有意に抑制し (P<0.05)、間質細胞における PGE2 に対する PGF の割合を有意に減少させた (P<0.05)。一方、上皮細胞において P4 は PGE2 合成を有意に刺激したが (P<0.05)、PGF 合成および *PLA2* ならびに *COX2* mRNA 発現には影響を及ぼさなかった。P4-BSA は子宮内膜上皮細胞における PGF および PGE2 合成を有意に刺激した が (P<0.05)、PGE2 に対する PGF の割合は変化しなかった。核内受容体の遺伝子発現は卵胞期において黄体期 (初期から中期) と比較し有意に高かった (P<0.05)。一方、膜受容体は排卵周期を通じて発現していたものの、その発現量に変化は認められなかった。

以上のことから、P4 は間質細胞において核内受容体を介して PGs 合成酵素発現を抑制することで PGs 合成を阻害する一方、上皮細胞において膜受容体を介して PGs 合成を促進することが示された。P4 は細胞種ごとに相反する作用を示し、子宮内膜における PGs 合成を厳密に制御することが明らかとなった。

(2)ウシ子宮内膜における排卵周期を通じた cortisol の PGF 抑制作用を調べる目的で上記の子宮内膜各期における GC receptor α (GC-Rα) タンパク質発現量を測定した。受容体の発現量と cortisol の作用の相関を調べるために黄体中期および卵胞期の子宮内膜組織に 10 nM cortisol を添加し 4 時間培養後の上清中 PGF 濃度を測定した。GC-Rα 発現の制御因子を調べる目的で培養間質細胞に cortisol (1, 10, 100 nM)、P4 (0.1, 1, 10 nM)、E2 (0.1, 1, 10 nM) を添加した。24 時間後に細胞を回収し GC-Rα mRNA およびタンパク質発現量を測定した。

ウシ子宮内膜の GC-Rα タンパク質発現量は黄体中期および後期において他の周期と比較して有意に高かった (P<0.05)。10 nM cortisol は黄体中期における PGF 合成を有意に抑制したが (P<0.05)、卵胞期ではその作用は見られなかった。子宮内膜間質細胞において cortisol は GC-Rα mRNA およびタンパク質発現を有意に抑制した (P<0.05)。一方 P4 は GC-Rα mRNA およびタンパク質発現を有意に刺激した (P<0.05)。E2 は GC-Rα の mRNA 発現に影響を及ぼさなかったがタンパク質発現を有意に抑制した (P<0.05)。

以上のことから、ウシ子宮内膜における cortisol の PGF 抑制作用は、黄体中期の子宮内膜における GC-Rα の発現と相関し、その発現は P4 により促進されることが示された。

本研究により、P4 および cortisol が子宮内膜における PGs 合成の重要な制御因子であることが示された。P4 は子宮内膜上皮細胞において膜受容体を介して PGF および PGE2 合成を刺激する一方で、間質細胞において核内受容体を介して PGF および PGE2 合成を抑制し、子宮内膜細胞全体で PGE2 合成優

位にシフトさせた。Cortisol は P4 によって発現増加する GC-Rα と結合することによって黄体中期に強い PGF 抑制作用を示した。以上のことから、黄体中期の子宮内膜において P4 および cortisol は PGF 合成を抑制し黄体を保護する働きを担うことが示された。