Regulation of prostaglandin biosynthesis by interleukin-1 in cultured bovine endometrial cells

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Abstract

Interleukin-1 (IL1) has been shown to be a potent stimulator of prostaglandin (PG) production in bovine endometrium. The aim of the present study was to determine the cell types in the endometrium (epithelial or stromal cells) responsible for the secretion of PGE2 and PGF2 α in response to IL1A, and the intracellular mechanisms of IL1A action. Cultured bovine epithelial and stromal cells were exposed to IL1A or IL1B (0.006–3.0 nM) for 24 h. IL1A and IL1B dose-dependently stimulated PGE2 and PGF2 α production in the stromal cells, but not in the epithelial cells. The stimulatory effect of IL1A (0.06–3.0 nM) on PG production was greater than that of IL1B. The stimulatory actions of IL1A on PG production was augmented by supplementing arachidonic acid (AA). When the stromal cells were incubated with IL1A and inhibitors of phospholipase (PL) C or PLA2 (1 μ M; anthranilic acid), only PLA2 inhibitor completely stopped the stimulatory action of

IL1A on PG production. Moreover, a specific cyclooxygenase-2 (COX2) inhibitor blocked the stimulatory effect of IL1A on PG production. IL1A (0.06 nM) promoted COX2 and microsomal PGE synthase-1 (*PGES1*) gene and its protein expression. The expression of COX1, *PGES2*, *PGES3*, and PGF synthase (*PGFS*) mRNA was not affected by IL1A in the stromal cells. The overall results indicate that 1) the target of IL1A and IL1B for stimulating both PGE2 and PGF2 α production is the stromal cells, 2) IL1A is a far more potent stimulator than IL1B on PG production in stromal cells, 3) the stimulatory effect of IL1A on PG production is mediated via the activation of PLA2 and COX2, and (4) IL1A induced PG production by increasing expressions of COX2 and *PGES1* mRNAs and their proteins in bovine stromal cells.

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Introduction

The cyclic nature of the bovine estrous cycle depends on the uterine prostaglandin F2 α (PGF2 α) and its action on the corpus luteum (Okuda *et al.* 2002). PGF2 α is well recognized as the main luteolytic agent in ruminants, whereas PGE2 (PGE2) has luteotropic and luteoprotective properties (Reynold *et al.* 1981, Weems *et al.* 1998). Although both PGE2 and PGF2 α are secreted by bovine endometrium throughout the estrous cycle (Miyamoto *et al.* 2000, Murakami *et al.* 2001), the relative proportion of PGE2 and PGF2 α secretion is thought to be more important than the absolute amounts of each PG to exert their own physiological effects on the female reproductive function (Murakami *et al.* 2001). Moreover, we have recently shown that PG production by bovine endometrium is differently regulated by interleukin-1 α (IL1A) throughout the estrous cycle (Tanikawa *et al.* 2005), suggesting that IL1A plays roles in the

local control of PGE2 and PGF2 α production in bovine endometrium during the estrous cycle. IL1B, which is an isomer of IL1A with action through the same receptor and similar biological activity (Dinarello 1988, 1991), also stimulated PG synthesis in bovine endometrial stromal and epithelial cells (Betts & Hansen 1992, Davidson *et al.* 1995). In addition, the presence of IL1B in bovine reproductive tract has been demonstrated by immunohistochemistry (Paula-Lopes *et al.* 1999). Thus, IL1A and IL1B appear to be important in regulating bovine endometrial function. However, the mechanisms involved in the differential effects of both IL1 on PGE2 and PGF2 α production remains to be unknown.

The bovine endometrium is a complex tissue and consists mainly of epithelial and stromal cells (Fortier *et al.* 1988). Endometrial epithelial cells have been demonstrated to synthesize mainly PGF2 α , whereas endometrial stromal cells synthesize approximately ten times more luteotropic PGE2

than epithelial cells (Kim & Fortier 1995, Asselin *et al.* 1996, Skarzynski *et al.* 2000). Therefore, it is also important to investigate the specific action of IL1A and IL1B on endometrial epithelial and stromal cells in order to understand the local control of PGE2 and PGF2 α production in bovine endometrium by IL1A and IL1B.

PGs are produced from arachidonic acid (AA) liberated from phospholipid stores through the action of phospholipases (PLs; Burgoyne & Morgan 1990). In bovine luteal cells (Townson & Pate 1994), IL1B appears to require the stimulation of PL and/or the metabolism of AA for the production of PGs. Moreover, in the bovine uterus, cyclooxygenase-2 (COX2) converts AA to PGH2 (Asselin *et al.* 1997), which is then converted by PGE synthase (PGES) and PGF synthase (PGFS) to PGE2 and PGF2 α respectively (Madore *et al.* 2003, Parent & Fortier 2005). Recently, the existence of at least three different forms of PGES (PGES1, PGES2, and PGES3) have been reported; among them, microsomal PGES-1 (PGES1) was found to be the main enzyme associated with cytokine-induced PGE2 production in the bovine endometrium *in vitro* (Parent & Fortier 2005). This enzyme is highly inducible along with COX2 (Madore *et al.* 2003, Parent & Fortier 2005). PGFS has recently been identified in bovine endometrium (Madore *et al.* 2003, Woclawek-Potocka *et al.* 2005). Since IL1A regulates the PGE2:PGF2 α ratio in bovine endometrium (Tanikawa *et al.* 2005), and IL1B stimulates PG synthesis in bovine endometrial cells (Betts & Hansen 1992, Davidson *et al.* 1995), we hypothesized that IL1A and IL1B have the ability to increase the production of endometrial PGE2 and PGF2 α by modulating key enzymes directly involved in PG biosynthesis pathways.

To address the above hypothesis, we investigated 1) the effect of IL1A and IL1B on PGE2 and PGF2 α synthesis in cultured bovine endometrial epithelial and stromal cells, especially focusing on 2) the effects of IL1 on expression of key enzymes directly involved in PG biosynthesis such as COX, PGES, and PGFS. 3) The intracellular mechanisms involved in IL1 action on PG synthesis via PL A2 and PL C were also studied.

Materials and Methods

Isolation of endometrial cells

Uteri of Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local institutional animal care and use committee. The estrus was synchronized using implants of a progesterone analogue (Crestar, Intervet, Holland), with additional injection of an analogue of PGF2 α (cloprostenol; Bioestrophon, Biowet, Gorzow Wielkopowski, Poland), as recommended by the manufacturer for the estrus synchronization of multiparous cows (Bah *et al.* 2006). In this study, uteri of the early luteal phase (days 2–5) were used. Uteri were obtained within 30 min after exsanguination and were transported to the laboratory within 1–1.5 h on ice. The epithelial and stromal cells from the bovine endometrium were

separated using procedures described previously (Skarzynski *et al.* 2000). A polyvinyl catheter was inserted into the side of the oviduct, and the ends of the horn were tied in order to retain trypsin solution for detaching the epithelial cells as described below. The uterine lumen was washed thrice with 30–50 ml of sterile Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (HBSS) supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 0.1% (w/v) BSA (Roche Diagnostics). Thirty to fifty milliliters of sterile HBSS containing 0.3% (w/v) trypsin (Sigma) was then infused into the uterine lumen through the catheter. Epithelial cells were isolated by incubation at 38 °C for 60 min with gentle shaking.

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 mm³). The minced tissues (~ 5 g) were digested by stirring for 60 min in 50 ml of sterile HBSS containing 0.05% (w/v) collagenase (Sigma), 0.005% (w/v) DNase I (Sigma), and 0.1% (w/v) BSA. The dissociated cells were filtered through metal meshes (100 μ m and 80 μ m) to remove undissociated tissue fragments. The filtrate was washed thrice by centrifugation (10 min at 100 g) with DMEM (Sigma) supplemented with antibiotics and 0.1% (w/v) BSA. After the washes, the cells were counted with a hemocytometer. The cell viability was higher than 85% as assessed by 0.5% (w/v) Trypan blue dye exclusion.

Culture of endometrial cells

The final pellet of the isolated epithelial or stromal cells were resuspended in culture medium (DMEM/Ham's F-12; 1:1 (v/v); Sigma) supplemented with 10% (v/v) calf serum (CS; Sigma), 20 μ g/ml gentamicin (Invitrogen), and 2 μ g/ml amphotericin B (Sigma; Skarzynski *et al.* 2000). The stromal cells were seeded at a density of 1×10^5 viable cells/ml in 48-well cluster dishes (Greiner Bio-One, Frickenhausen, Germany) for experiments 1 and 2, in 24-well cluster dishes (Costar, Cambridge, MA, USA) for experiment 3, or in 80 cm² culture flask (Greiner Bio-One) for experiment 4, and the epithelial cells were seeded at a density of 1×10^5 viable cells/ml in culture flasks (Nunc) and cultured at 38 °C in a humidified atmosphere of 5% CO₂ in air. To purify the stromal preparation, the medium was changed 2 h after plating, by which time selective attachment of stromal cells had occurred (Fortier *et al.* 1988, Skarzynski *et al.* 2000). Alternatively, since the epithelial cells attached 24–48 h after plating, the medium was changed every 2 days until the cells reached confluence. When the epithelial cells were confluent, 0.02% (w/v) trypsin solution was added to the cells to remove the other cells. After removal of the other cells, 0.25% (w/v) trypsin solution was then added to the epithelial cells to collect the pure epithelial cells. The cells removed, adjusted to a density of 1×10^5 cells/ml, and placed in

48-well cluster dishes for DNA quantification in fresh DMEM/Ham's F-12 supplemented with 10% (w/v) CS, 20 µg/ml gentamicin, and 2 mg/ml amphotericin B until the cells reached confluence. The homogeneity of stromal cells and epithelial cells was evaluated using immunofluorescent staining for specific markers of epithelial (cytokeratin) and stromal cells (vimentin) as described previously (Malayer & Woods 1998). The epithelial cell contamination of stromal cells was about 1% and stromal cells contamination of epithelial cells <1% (Fig. 1). These values were similar to the results of our previous study (Lee *et al.* 2007). When cells of each type 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 sodium selenite (Sigma), 0.5 mM ascorbic acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 5 µg/ml transferrin (Sigma), 2 µg/ml insulin (Sigma), and 20 µg/ml gentamicin. The cells were then exposed to different concentrations of IL1A for 24 h.

Experiment 1 To determine the dose-dependent effects of IL1A and IL1B on PGE2 and PGF2 α production in the bovine epithelial and stromal cells, the cells were exposed to IL1A (0.006–3.0 nM; Dainippon Pharmaceutical Co., Ltd., Osaka,

Japan) or IL1B (0.006–3.0 nM; PeproTech House Co., London, UK) for 24 h. Oxytocin (OT, 100 nM; Teikoku Hormone MFG Co., Tokyo, Japan) and tumor necrosis factor- α (TNF, 0.06 nM; Dainippon Pharmaceutical Co., Ltd) was used. The concentrations of OT and TNF were based on a previous study (Skarzynski *et al.* 2000). We previously reported that TNF stimulates both PG productions in stromal cells but not in epithelial cells (Skarzynski *et al.* 2000). Thus, TNF was used as a positive control to confirm that endometrial stromal cells were responsive in the present culture system.

Experiment 2 To determine the intracellular mechanisms involved in IL1A actions on PG synthesis by the bovine stromal cells, the cells were exposed to a PLA2 inhibitor (anthranilic acid, ACA; 1 µM; Calbiochem, San Diego, CA, USA), a PLC inhibitor (1 µM; U-73122, Calbiochem; #662035), AA (10 µM; Sigma), or a selective COX2 inhibitor (5 nM; NS-398; BIOMOL, Plymouth Meeting, PA, USA) with IL1A (0.06 nM) or without for 24 h.

After culture, in experiment 1 and experiment 2, the conditioned medium was collected in tubes with 5 µl 0.3 M EDTA, 1% (w/v) aspirin (Sigma) solution (pH 7.3), and frozen at –30 °C until the PG assay. The DNA content of the

Figure 1 Representative photomicrograph of immunostaining for vimentin and cytokeratin in endometrial (A) stromal and (B) epithelial cells. The cells were incubated with primary antibody against either vimentin (monoclonal anti-vimentin; 1:200 dilution; upper and lower middle) or cytokeratin (monoclonal anti-cytokeratin peptide; 1:100 dilution; upper and lower right), then with second antibody (anti-mouse IgG FITC conjugate-donkey; 1:200 dilution), and were visualized by fluorescence microscopy. Controls were prepared in the absence of primary antibody (upper and lower left).

epithelial and stromal cells was estimated spectrophotometrically as described by Labarca & Paigen (1980), to standardize the results. Briefly, after collection of the media, the cells were washed two times with 500 µl of phosphate-saline buffer (50 mM NaH₂PO₄ 2H₂O, 140 mM NaCl, 10 mM NaH₂PO₄, 2 mM EDTA; pH 7·4) and ultrasonicated for 20 s. The samples and standard (100 µl) were dispensed in a 96-well plate (Griner Bio-One), and then 40 µl of bisbenzimidazole (8·43 µM; Sigma) was added into each well of the plate. After a 10-min incubation at 4 °C, fluorescence was evaluated using a Plate reader, Fluorescence II (Flow Laboratories). The DNA from the calf thymus (Sigma) was used as a standard, and the standard curve was determined for concentrations in the range from 0·1 to 20 µg/ml. Since IL1A stimulates PGE2 and PGF2α production in stromal cells, but not in epithelial cells, experiments 3 and 4 were performed only in stromal cells.

Experiment 3 To determine the effect of IL1A on mRNA expressions of enzymes directly involved in PG production such as COX1, COX2, PGES1, PGES2, PGES3, and PGFS in bovine stromal cells, cells were exposed to IL1A (0·06 nM) for 24 h, disrupted with 1 ml of TRIZOL Reagent (Invitrogen), and frozen at -80 °C until determination of the mRNA.

Experiment 4 To determine the effect of IL1A on COX2, PGES1, and PGFS protein expression in bovine stromal cells, the cells were exposed to IL1A (0·06 nM) for 24 h. The cultured cells were scraped and placed in ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete (protease inhibitor cocktail; Roche), pH 7·4), then frozen in liquid nitrogen, and stored at -80 °C until protein analysis by western blotting.

PGE2 and PGF2α determination

The concentrations of PGE2 and PGF2α in the culture media were determined with an enzyme immunoassay described previously (Uenoyama *et al.* 1997, Skarzynski & Okuda

2000). The PGE2 standard curve ranged from 0·039 to 10 ng/ml, and the ED50 of the assay was 0·625 ng/ml. The intra- and interassay coefficients of variation were on average 10·6 and 7·4% respectively. The crossreactivities of the anti-PGE2 serum, validated by comparing the inhibition of binding of peroxidase-labeled PGE2 to antiserum, were as follows: PGE2, 100%; PGE1, 15%; PGJ2, 14%; PGA1, 10%; PGB2, 6·7%; PGA2, 4·6%; PGF2α, 2·8%; PGD2, 0·13%; and 15-keto-PGE2, 0·05%. The PGF2α (Sigma) standard curve ranged from 0·016 to 4 ng/ml, and the ED50 of the assay was 0·25 ng/ml. The intra- and interassay coefficients of variation were 7·3 and 11·2% respectively.

RNA isolation and cDNA synthesis

Total RNA was prepared from cultured endometrial cells using TRIZOL Reagent according to the manufacturer's directions (Invitrogen; no. 15596-026). Total RNA (1 µg) was reverse transcribed using a ThermoScript RT-PCR System (Invitrogen).

Real-time PCR

Gene expression was measured by real-time PCR using the Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) and QuantiTect SYBR Green PCR system (Qiagen GmbH) starting with 2 ng of reverse-transcribed total RNA. For quantification of the mRNA expression levels, the primer length (20 bp) and GC contents of each primer (50–60%) were selected (Table 1), and standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1000). Expression of GAPDH was used as an internal control. 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's correlation coefficient; *r* > 0·99). Each

Table 1 Primers used in real time

Gene	Primer	Sequence	Accession numbers	Product (bp)
COX1	Forward	5'-CTGGGAGTCCTTCTCCAATG-3'	AF004943	101
	Reverse	5'-GCAACTGCTTCTCCCTTTG-3'		
COX2	Forward	5'-TGTGAAAGGGAGGAAAGAGC-3'	AF004944	115
	Reverse	5'-GGCAAAGAATGCAAACATCA-3'		
PGES1	Forward	5'-AGGACGCTCAGAGACATGGA-3'	AY032727	142
	Reverse	5'-TTCGGTCCGAGGAAAGAGTA-3'		
PGES2	Forward	5'-CCTACAGAAAGGTGCCCATC-3'	AY692441	109
	Reverse	5'-TGCCCTGACACCAGATAGGT-3'		
PGES3	Forward	5'-AAGGAGAATCTGGCCAGTCA-3'	AY692440	106
	Reverse	5'-TCGGAATCATCTTCCAGTC-3'		
PGFS	Forward	5'-TGGCCTCAACAGCAATATGA-3'	D88749	102
	Reverse	5'-TGCACGTGTACAGCTCAGC-3'		
GAPDH	Forward	5'-CACCCTCAAGATTGTCAGCA-3'	BC102589	103
	Reverse	5'-GGTCATAAGTCCCCTCACGA-3'		

PCR product was sequenced to ensure that the correct mRNA–cDNA was amplified. In addition, dissociation curve analysis was run following each real-time experiment to confirm the presence of only one product and the absence of the formation of primer dimers. The amplification efficiency rate for amplicons averaged over 99%. Relative concentrations of products are reported as the ratio of the target cDNA amplicon to the DAPDH amplicon, which was used as a loading control.

COX2, PGES1, and PGFS protein analysis

COX2, PGES1, and PGFS proteins level in cultured bovine endometrial stromal cells was assessed by western blotting analysis. The cultured cells (experiment 4) were ultrasonicated on ice and lysed in 100 µl of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol (Sigma), 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 (50 mM Tris–HCl, 2% SDS (Nacalai Tesque, Inc., Kyoto, Japan), 10% glycerol, 1% β-mercaptoethanol (Wako Pure Chemical Industries), pH 6.8), and heated at 95 °C for 10 min. Samples (50 µg protein) were subjected to electrophoresis on a 10% (COX2 and PGFS) or 15% (PGES1) SDS-PAGE for 1 h at 200 V. The separated proteins were electrophoretically transblotted to a 0.2 µm nitrocellulose membrane (Invitrogen; for COX2 and PGFS) or polyvinylidene difluoride membrane (Invitrogen) for PGES1 at 250 mA for 3 h in transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was then washed in TBS-T (0.1% Tween 20 in TBS (25 mM Tris–HCl, 137 mM NaCl, pH 7.5)), and cut into two pieces: one piece was used for a target protein (COX2 (72 kDa), PGES1 (16 kDa), PGFS (36.7 kDa)) and another piece was used for GAPDH (internal standard; 40 kDa). The pieces for each protein were incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at 4 °C. After the blocking incubation, the pieces of the membrane were separately incubated with a primary antibody specific to each protein. COX2 antibody (Alpha Diagnostic, San Antonio, TX, USA; 1:2000), PGES1 antibody (Cayman, Ann Arbor, MI, USA; 1:1000), PGFS antibody (kindly donated by Dr Watanabe, Division of Applied Life Sciences, Graduate School of Integrated Sciences and Arts, University of East Asia, Shimonoseki, Yamaguchi, Japan; 1:2000), and GAPDH antibody (Lab Frontier, Seoul, Korea; 1:10 000) in TBS-T, overnight at 4 °C. After incubation, the membrane pieces were washed thrice for 10 min in TBS-T at room temperature, and then incubated with secondary antibody (anti-rabbit Ig, HRP-linked whole antibody produced in goat, Assay Designs Inc., MI, USA; 1:10 000 in TBS-T) for 1 h, and washed thrice in TBS-T for 10 min at room temperature. The signal was detected by pico EPD (enhanced peroxidase detection) Western Blot Detection kit (Mbiotech, Seoul, Korea). The intensity of the immunological reaction in the cells was

estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, USA).

Statistical analysis

The experimental data are shown as the mean ± S.E.M. of values obtained from three to four separate experiments. Each experiment was performed in triplicate using endometrial cells obtained from a different cow. The level of PGF2α or PGE2 production (measured in culture media) was normalized to DNA concentrations and expressed as ng/µg DNA. The statistical significance of differences between controls and treated group in production of both PGs were assessed by ANOVA followed by a Fisher's protected least-significant difference procedure as a multiple comparison test. The statistical significance of differences in the relative amounts of mRNA and proteins was determined using the ratio of the target cDNA amplicon to the GAPDH amplicon and that of the target protein to GAPDH protein respectively, by ANOVA followed by Student's *t*-test.

Results

Dose-dependent and cell type-specific effects of IL1A and IL1B on PG production

IL1A and IL1B dose-dependently stimulated both PGE2 and PGF2α production in stromal cells ($P < 0.05$), whereas the stimulatory effects of both IL1s were not observed in epithelial cells (Fig. 2). The stimulatory effect of IL1A (0.06–3 nM) on both PG production was greater than that of IL1B (Fig. 3A and B). TNF and OT stimulated the production of both PGE2 and PGF2α in stromal cells and epithelial cells respectively ($P < 0.05$), which accounts for appropriate response of the cells in the present culture system. In contrast to IL1A, OT significantly increased both PGE2 and PGF2α production in epithelial cells ($P < 0.05$), which also accounts for the appropriate response of the epithelial cells. Since the stimulatory effect of IL1A on PG production was clearly greater than that induced by the same dose of IL1B in stromal cells, IL1A was used to further investigate the intracellular mechanisms involved in PG stimulation in endometrial stromal cells.

Effects of PL inhibitors, AA, and COX2 inhibitor on IL1A-induced PG production in stromal cells

The basal production of both PGs was not influenced by a PLA2 inhibitor (ACA) in stromal cells (Fig. 4). Although a PLC inhibitor (U-73122) showed no significant effects on basal and IL1A-induced PG production, ACA completely blocked the stimulatory action of IL1A ($P < 0.05$). Arachidonic acid increased basal PG production in stromal cells. Moreover, the stimulatory effect of IL1A on production of

Figure 2 Effects of (A and C) IL1A and (B and D) IL1B on PGE2 (white bars) and PGF2α (gray bars) production by (A and B) cultured bovine stromal and (C and D) epithelial cells. IL1A or IL1B (0.006–3.0 nM) was added 24 h before the end of culture. Experiments with isolated cells were performed three times each with separate cell preparations. Data are the mean $\pm$ S.E.M. of three separate experiments. Different superscript letters *a, b, c* (PGE2) and *x, y, z* (PGF2α) indicated significant differences ($P < 0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

both PGs was augmented by AA ($P < 0.001$). IL1A-induced production of PG was blocked by a specific COX2 inhibitor (NS-398; $P < 0.05$), whereas NS-398 showed no significant effect on basal PG production (Fig. 4).

Effects of IL1A on COX1, COX2, PGES1, PGES2, PGES3, and PGFS gene expressions in stromal cells

IL1A (0.06 nM) increased the mRNA expressions of COX2 (Fig. 5B) and PGES1 (Fig. 5C), whereas IL1A did not affect the expression of COX1, COX2, PGES3, and PGFS mRNA in stromal cells (Fig. 5A, D and F).

Effects of IL1A on COX2, PGES1, and PGFS protein levels in stromal cells

Specific bands of COX2, PGES1, and PGFS were expressed in stromal cells (Fig. 6A–C), in agreement with a previous result (Woclawek-Potocka *et al.* 2005). Intensities of the bands, after normalization to GAPDH-specific bands, showed that IL1A (0.06 nM) increased COX2 and PGES1

protein expression in stromal cells (Fig. 6A and B; $P < 0.05$). PGFS protein expression in bovine stromal cells was not stimulated by IL1A (Fig. 6C).

Discussion

The present study demonstrated that IL1A stimulates PGE2 and PGF2α production in the stromal cells, but not in the epithelial cells of the bovine endometrium, and that PLA2 and COX2 are directly involved in the stimulatory actions of IL1A on both PGE2 and PGF2α production. These findings suggest that IL1A regulates the production of bovine uterine PGE2 and PGF2α, mainly in endometrial stromal cells by regulating key enzymes involved in PG biosynthesis.

IL1B is an isomer of IL1A that acts through the same receptor as IL1A and has similar biological activity (Dinarello 1988, 1991). IL1B stimulated the production of PG in endometrial stromal and epithelial cells isolated from bovine endometrium exposed to high levels of progesterone on day 16 of pregnancy (Betts & Hansen 1992) and on day 11 or 14 of the estrous cycle

Figure 3 Effect of IL1A (○) and IL1B (●) on (A) PGE2 and (B) PGF2α production in cultured bovine endometrial stromal cells. IL1A and IL1B (0.006–3 nM) were added 24 h before the end of culture. Asterisks indicate a significant difference in PG production between cells treated with the same dose of IL1A and IL1B, as determined by ANOVA followed by Student's *t*-test. Different superscript letters: *a, b, c* (IL1A) and *x, y, z* (IL1B) indicate significant differences between treated and untreated cells ($P < 0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

(Davidson *et al.* 1995). However, the effects of IL1A on PG production were not analyzed. Using endometrial cells that were isolated from the uterus between days 2 and 5 after estrus, and that were exposed to high levels of estradiol, we demonstrated that IL1 has a cell type-specific action affecting PG production in stromal cells, but not in epithelial cells. The discrepancy between the results of the present and previous studies may be due to the different time of exposure to estradiol (present study) and progesterone (former studies). However, in the preliminary experiment, we found that the stage of the estrous cycle has a minimum effect on the secretory response of endometrial cells in the present culture system. In addition, the human recombinant IL1A and IL1B used in the present study dose-dependently increased PGF2α and PGE2 production by

Figure 4 Effects of ACA (a PLA2 inhibitor), U-73122 (a PLC inhibitor) AA and NS-398 (a COX2 inhibitor) on IL1A-stimulated (A) PGF2α and (B) PGE2 production by stromal cells. ACA (1 μM), U-73122 (1 μM), AA (10 μM), NS-398 (5 nM), and IL1A (0.06 nM) were added 24 h before the end of culture. Experiments with isolated cells were performed three times each with separate cell preparations. Data are the mean $\pm$ s.e.m. of three separate experiments. Different superscript letters indicate significant differences ($P < 0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

the luteal cells of all stages in our previous study (Nishimura *et al.* 2004). Thus, the differences in the cell-specific response of cultured bovine endometrial cells to IL1A and IL1B were not due to differences in the specific activity of the human recombinant IL1. Since the stimulatory effect of IL1A on PG was clearly greater than that induced by the same dose of IL1B in stromal cells, IL1A was used to further investigate the intracellular mechanisms involved in PG stimulation in endometrial stromal cells.

IL1 activates cellular responses by interacting with a signaling receptor complex formed by a functional receptor, IL1R type I (IL1RT1). IL1R type II (IL1RT2), a decoy receptor acts as a negative pathway of regulation of IL1, can more efficiently bind to IL1B than IL1A (Arend 1991, Colotta *et al.* 1994, Boraschi *et al.* 1996). Thus, the results of the present and previous studies raise the possibility that IL1A exerts a more potent effect than the same dose of IL1B due to a decrease in the amount of IL1B which can bind IL1RT1 in

Figure 5 Effects of IL1A on the amounts of (A) COX1, (B) COX2, (C) PGES1, (D) PGES2, (E) PGES3, and (F) PGFS mRNA expression in cultured bovine stromal cells. IL1A (0.06 nM) was added 24 h before the end of culture. Experiments with isolated cells were performed three times each with separate cell preparations. Data are the mean $\pm$ S.E.M. of three separate experiments, and are expressed as the relative levels to GAPDH mRNA levels. Asterisk indicates significant differences ($P < 0.05$), as determined by ANOVA followed by Student's *t*-test.

the stromal cells. However, since the expression of IL1RT2 in bovine endometrium is still unclear, further studies are needed to clarify these points.

PGs are produced from AA liberated from phospholipid stores through the action of PLs (Burgoyne & Morgan 1990). In the present study, an inhibitor of PLA2 (ACA) completely stopped the stimulatory action of IL1A, whereas an inhibitor of PLC (U-73122) did not significantly affect IL1A-induced PG production. The failure of IL1A action on stimulating PG production in the stromal cells treated with ACA may be due to a lower accumulation of AA. This supposition is supported by the fact that exogenous AA strongly augmented IL1A-stimulated PG production, suggesting that IL1A influences the downstream of AA cascade metabolized by PLA2 in the PG biosynthesis pathways.

COX2 is an inducible key rate-limiting enzyme for converting AA to the unstable form of PGs, PGG2/PGH2, which is the first step in the synthesizing of PGs (Morita 2002,

Figure 6 Effects of IL1A on COX2, PGES1, and PGFS protein levels in cultured bovine stromal cells. IL1A (0.06 nM) was added 24 h before the end of culture. Experiments with isolated cells were performed three times each with separate cell preparations. Representative samples of western blot for (A) COX2, (B) PGES1, (C) PGFS and GAPDH are shown in upper panels. Data are the mean $\pm$ S.E.M. of four separate experiments, and are expressed as the relative ratio of (A) COX2, (B) PGES1, (C) PGFS protein to GAPDH protein. Asterisk indicates significant differences ($P < 0.05$) among treatments, as determined by ANOVA followed by Student's *t*-test.

Tanabe & Tohna 2002). In the present study, IL1A stimulated COX2 expressions and increased the production of both PGs in bovine endometrial stromal cells. Moreover, a specific inhibitor of COX2 (NS-398) blocked IL1A-stimulated PG synthesis in the stromal cells. These findings suggest that the increase in both PGF2 α and PGE2 production induced by IL1A is due to increasing COX2 expression and activating this enzyme.

The bovine endometrium has been shown to express three different isoforms of PGE synthase (PGES1, PGES2, and PGES3). PGES1 seems to be the main PGE synthase responsible for increasing PGE2 production in endometrial cells (Parent & Fortier 2005). In the present study, although IL1A increased *PGES1* mRNA and protein expression concomitant with COX2, it did not affect *PGES2* and *PGES3* mRNA. Thus, IL1A seems to stimulate PGE2 biosynthesis in bovine endometrial stromal cells via stimulating inducible COX2 and PGES1 expression.

Some genes have been brought as candidates for regulating PGF2 α synthesis in bovine endometrial cells. Madore *et al.* (2003) showed that 20 α -hydroxysteroid dehydrogenase (AKR1B5) possesses potent PTGFS activity. Furthermore, they showed another gene expression that is related to PGF2 α production, PGF2 α -like 2 synthase (PGFSL2). The specific stimulatory effects of OT in endometrial epithelial cells and TNF in endometrial stromal cells on PGF2 α production have also been demonstrated to be caused via an increase in PGFSL2 gene and protein expressions (Woclawek-Potocka *et al.* 2005). However, in the present study, IL1A did not affect the mRNA expression of *AKR1B5* and *PGFSL2* in bovine stromal cells (data not shown). Then, we focused on *bovine liver-type PGFS*, which cDNA (GenBank accession no. D88749) shared 94% identity with bovine *PGFSL2* (GenBank accession no. AY135401) and both genes belong to the AKRC1 family (Madore *et al.* 2003). Though in the present study, the expression of *PGFS* mRNA and protein was detected, the stimulation effect of IL1A on *PGFS* gene and protein expression was not observed in stromal cells in spite of that IL1A-induced PGF2 α production was demonstrated in stromal cells. Thus, it is thought that IL1A induced PGF2 α production is only via COX2.

The overall results indicate that (1) the target of IL1A and IL1B for stimulating both PGE2 and PGF2 α production is the stromal cells, (2) IL1A is far more potent stimulator than IL1B on PG production in stromal cells, (3) the stimulatory effect of IL1A on PG production is mediated via the activation of PLA2 and COX2, and (4) IL1A induced PG production by increasing expressions of COX2 and *PGES1* mRNAs and their proteins in bovine stromal cells.

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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