Expressions of Estrogen Receptors in the Bovine Corpus Luteum: Cyclic Changes and Effects of Prostaglandin F2α and Cytokines

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Abstract. Estrogen (E) exerts its function by binding to two intracellular estrogen receptors, ERα and ERβ. Although ERs have been reported to be expressed in the bovine corpus luteum (CL), the mechanisms that control ER expression in the bovine CL are not fully understood. To determine the possible regulatory mechanisms of ERα and ERβ that mediate distinct E functions, we examined 1) the changes in the protein expressions of ERs in the CL throughout the luteal phase and 2) the effects of prostaglandin (PG) F2α, tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) on the expressions of ERs in cultured bovine luteal cells. Western blot analyses revealed that ERα and ERβ proteins were expressed throughout the luteal phase. The ERα protein level was high at the early luteal (Days 2–3 after ovulation) and mid-luteal stages (Days 8–12) and was extremely low at the regressed luteal stage (Days 19–21). The ERβ protein level increased from the early to developing luteal stage, remained at the same level at the mid-luteal stage and decreased thereafter. The ratio of ERβ to ERα was higher in the regressed stage than in the other stages. Luteal cells obtained from mid-stage CLs (Days 8–12) were incubated with PGF2α (0.01–1 µM), TNFα (0.0145–1.45 nM) or IFNγ (0.0125–1.25 nM) for 24 h. PGF2α and TNFα inhibited ERα and ERβ mRNA expressions. IFNγ suppressed ERβ mRNA expression but did not affect the expression of ERα mRNA. However, the ERα and ERβ protein levels were not affected by any of the above treatments. These data indicate that PGF2α, TNFα and IFNγ regulate ERα and ERβ mRNA expressions in bovine luteal cells. Moreover, the changes in the ERβ/ERα ratio throughout the luteal phase suggest that ERα is associated with luteal maintenance. Therefore, a dramatic decrease in ERα at the regressed luteal stage could result in progression of structural luteolysis in the bovine CL.

Key words: Cattle, Corpus luteum, Cytokines, Estrogen receptor, Prostaglandin F2α

Estrogen (E) is involved in secretory function, cell differentiation and cell death in female reproductive organs [1–3]. E is mainly synthesized and secreted by the granulosa cells of growing and preovulatory follicles [4]. E production and the mRNA of the E receptor (ER) in the bovine corpus luteum (CL) change throughout the luteal phase [5–8]. Therefore, it is possible that E plays a role as a paracrine/autocrine regulator of CL function throughout the luteal phase. However, the precise role of E in the bovine CL is not fully understood.

ER is a member of the nuclear receptor superfamily (transcription factors), whose members are activated by the binding of a ligand [9, 10]. It is
generally accepted that there are two different ER subtypes, i.e. ERα and ERβ [3, 7, 10, 11]. In the bovine CL, ER mRNA levels change significantly during the estrous cycle [7]. ERα mRNA expression is low at the late (Days 13–16 after ovulation) and regressed (Days 18–21) luteal stages [7]. However, the levels of ER protein throughout the luteal phase and the mechanisms that control ER expressions in luteal cells during luteal regression are still unknown.

Prostaglandin (PG) F2α, which is primarily secreted by the endometrium [12–14], is considered to be an important regulator of reproductive processes, including luteolysis in cattle [15]. Days 15–17 are considered to be a critical period in cattle at which luteolysis is initiated by an increase in uterine PGF2α secretion in the absence of a viable embryo [16]. During the estrous cycle, the PGF2α output from the bovine endometrium reaches its highest level at the regressed luteal stage and declines during the early and mid-luteal stages [17]. PGF2α induces a number of changes in gene expression related to regulation of luteal function [18–20]. Although PGF2α modulates ER mRNA expression in the porcine CL [20], whether PGF2α affects ER expressions in the bovine CL is unknown. Together with increased output of endometrial PGF2α, the numbers of leukocytes (e.g. T lymphocytes, macrophages) increase in CL at the time of luteolysis in cows [21]. Leukocytes produce a variety of cytokines, including tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ), which have been shown to affect luteal cell function in vitro [22–25]. Since TNFα modulates ER expressions in human MCF-7 breast cancer cells [26] and IFNγ upregulates TNFα receptors in a variety of cell types [27, 28], we hypothesized that cytokines regulate ER expressions in the bovine CL.

In the present study, we characterized the patterns of ERα and ERβ protein expressions throughout the luteal phase. To clarify the regulatory mechanisms of ER expressions in luteal cells, the effects of PGF2α, TNFα and IFNγ on the luteal ERα and ERβ expressions in bovine luteal cells were also investigated.

Materials and Methods

Collection of CLs

Ovaries were collected from Holstein cows at a local abattoir in accordance with protocols approved by the local Institutional Animal Care and Use Committee. Ovaries with CL were obtained within 10–20 min after exsanguination. Luteal stages were classified as early (Days 2–3 after ovulation), developing (Days 5–6), mid (Days 8–12), late (Days 15–17) and regressed (Days 19–21) by macroscopic observation of the ovary and uterus as described previously [17, 29]. After determination of the stages, the CLs (n=4/stage) were immediately separated from the ovaries, frozen rapidly in liquid nitrogen and stored at −80°C until being processed for protein analysis. For experiments involving cell culture, ovaries with CL were submerged in ice-cold physiological saline and transported to the laboratory.

ERα and ERβ protein analysis

CL tissues were homogenized on ice in homogenization buffer using a tissue homogenizer (Physcotron, Microtec, Chiba, Japan) and then filtered with a metal wire mesh (150 µm). The cultured cells were ultrasonicated on ice in the homogenization buffer. For ERα and ERβ protein analysis, nuclei were isolated from the tissue and cell homogenates by centrifugation at 600 × g for 30 min. The protein concentration was determined by the method of Osnes et al. [30] using BSA as a standard. Bovine caruncular endometrial tissue was used as a positive control.

The ERα and ERβ protein levels were assessed by Western blotting analysis. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% [w/v] SDS [No. 31607-94; Nacalai Tesque, Kyoto, Japan], 10% [v/v] glycerol, 1% [v/v] β-mercaptoethanol [No. 137-06862; Wako Pure Chemical, Osaka, Japan], pH 6.8) and heated at 95°C for 10 min. Samples (50 µg protein) were subjected to electrophoresis on a 15% SDS-PAGE for 1 h at 200 V. The separated proteins were electrophoretically transblotted onto a 0.45 µm nitrocellulose membrane (No. LC2001; Invitrogen, Carlsbad, CA, USA) at 60 V for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycerol and 20% [v/v] methanol, pH 8.3). The membrane was then washed in TBS (25 mM Tris-HCl, pH 7.5 and 137 mM NaCl) and incubated in blocking buffer (4% [w/v] nonfat dry milk in
TBS-T [0.1%; v/v Tween 20 in TBS] overnight at 4 C. After blocking incubation, the pieces of membrane were separately incubated in blocking buffer with a primary antibody specific to each protein: ERα antibody (No. sc 7207; Santa Cruz Biotechnology, Heidelberg, Germany; 1:100, 2 h at room temperature), ERβ antibody (No. PA1-311; Affinity BioReagents, Golden, CO, USA; 1:500, 2 h at room temperature) and β-actin antibody (No. A2228; Sigma-Aldrich, Co., St. Louis, MO, USA; 1:4,000, 75 min at room temperature). After incubation, the membrane pieces were washed three times for 10 min in TBS-T at room temperature, incubated in blocking buffer with a secondary antibody, donkey anti-rabbit Ig HRP-linked whole antibody (No. NA934; Amersham Biosciences, Piscataway, NJ, USA) for ERα (1:10,000) and ERβ (1:20,000) and sheep anti-mouse Ig HRP-linked whole antibody (No. NA931; Amersham Biosciences) for β-actin (1:40,000), for 45 min and then washed three times in TBS for 10 min at room temperature. The signal was detected using an ECL Western Blotting Detection System (No. RPN2109; Amersham Biosciences).

The intensity of the immunological reaction (ERα, ERβ and β-actin) in the tissues or cells was estimated by measuring the optical density in a defined area by computer-assisted densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

Cell isolation

Enzymatic dissociation of mid-luteal tissue and culture of luteal cells were performed as described previously [31]. The luteal cells were suspended in a culture medium, DMEM and Ham’s F-12 medium (No. D8900; 1:1 [v/v], DMEM/F-12, Sigma-Aldrich) containing 5% calf serum (No. 16170-078; Life Technologies, St. Louis, MO, USA) and 20 µg/ml gentamicin (No. G1397; Sigma-Aldrich). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small luteal cells, 20% large luteal cells and 10% endothelial cells or fibrocytes; no erythrocytes were present.

Cell culture

The dispersed luteal cells were seeded at 2.0 × 10⁶ viable cells per ml in 24-well cluster dishes (No. 3524; Costar, Cambridge, MA, USA) for total RNA extraction or an 80 cm² culture flask (No. 658175; Greiner Bio-One, Frickenhausen, Germany) for protein analysis. They were then cultured in a humidified atmosphere of 5% CO₂ in air at 37.5 C in a N₂-O₂-CO₂-regulated incubator (No. BNP-110; ESPEC, Osaka, Japan). After 18 h of culture, the medium was replaced with phenol red-free, serum-free fresh medium, DMEM/F-12 (No. D2906; Sigma-Aldrich) containing 0.1% [w/v] BSA, 5 ng/ml sodium selenite, 5 µg/ml transferrin and 2 µg/ml insulin. The following experiments were then carried out. The luteal cells for total RNA extraction were treated with 0.01, 0.1 or 1 µM PGF2α (No. P5069; Sigma-Aldrich), 0.0145, 0.145 or 1.45 nM recombinant human TNFα (kindly donated by Dainippon Pharmaceutical, Osaka, Japan) or 0.0125, 0.125 or 1.25 nM recombinant bovine IFNγ (kindly donated by Dr. Inumaru, National Institute of Animal Health, Ibaraki, Japan) for 24 h. All treatments were conducted in triplicate. After culture, the luteal cells were collected and stored at –80 C until total RNA extraction. The luteal cells for protein analysis were treated with PGF2α (1 µM) or TNFα (1.45 nM) and/or IFNγ (1.25 nM) for 24 h. The cultured cells were scraped, placed in ice-cold homogenization buffer [25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA and Complete Protease Inhibitor Cocktail (No. 1697498; Roche Diagnostics, Mannheim, Germany), pH 7.4], frozen in liquid nitrogen and stored at –80 C until ERα and ERβ protein analysis by Western blotting.

RNA isolation and cDNA synthesis

Total RNA was prepared from cultured luteal cells using TRIZOL Reagent according to the manufacturer’s directions (No. 15596-026; Invitrogen). Total RNA (1 µg) was reverse transcribed using a ThermoScript RT-PCR System (No. 11146-016; Invitrogen).

Real-time polymerase chain reaction (PCR)

Gene expression was measured by real-time PCR using a thermal cycler (No. 170-9770J1; MyiQ Single-Color Real-Time PCR Detection System; Bio-Rad Laboratories, Hercules, CA, USA) and the iQ SYBR Green Supermix (No. 170-8880; Bio-Rad) starting with 3.8 ng of reverse-transcribed total RNA. Standard curves of sample cDNA were generated using serial dilutions (1:2 to 1:1000). GAPDH expression was used as an internal control. Twenty-bp primers with 50–60% GC-contents were synthesized (Table 1). The PCR conditions were as
follows: 95°C for 15 min followed by 55 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Use of iQ SYBR Green Supermix at elevated temperatures resulted in reliable and sensitive quantification of the PCR products with high linearity (Pearson correlation coefficient: r>0.99).

### Statistical analysis
All experimental data are shown as means ± SEM. The statistical significance of differences in the amounts of ERα and ERβ mRNAs and the ERα and ERβ protein levels was assessed by analysis of variance (ANOVA) followed by Fisher’s protected least significant difference procedure (PLSD) as a multiple comparison test. The statistical significance of differences in the ERα and ERβ protein expressions between control and PGF2α-treated cells was assessed by Student’s t-test. The ratio of ERβ to ERα protein was the absolute level of ERβ/β-actin protein (measured in arbitrary units) divided by the absolute level of ERα/β-actin (measured in arbitrary units) in each experiment.

### Results

**ERα and ERβ protein expressions in the bovine CL throughout the luteal phase**

ERα and ERβ proteins were expressed through-

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### Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Accession no.</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>Forward</td>
<td>CAGGCACATGAGCAACAAAG</td>
<td>AY538775</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCAGCAGCGGTCTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>Forward</td>
<td>CTGAAGCAGAATCCAGCAC</td>
<td>Y18017</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGGAAGGACACATAGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CACCCCTAAGATTGTCAAGCA</td>
<td>BC102589</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTCAATAGTCCTCCAGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
out the luteal phase. The ERα protein level was higher at the early luteal and mid-luteal stages than at the other luteal stages and was lowest at the regressed luteal stage (Fig. 1A; P<0.05). The ERβ protein level increased from the early to developing luteal stage, remained at the same level at the mid-luteal stage and decreased thereafter (Fig. 1B). The ratio of ERβ to ERα was higher in the regressed stage than in the other stages (Fig. 1C; P<0.01).

**Effects of PGF2α, TNFα and IFNγ on the ERα and ERβ mRNA expressions in cultured bovine mid-luteal cells**

PGF2α and TNFα inhibited the expressions of ERα and ERβ mRNA (Figs. 2 and 3; P<0.05). IFNγ suppressed ERβ mRNA expression (Fig. 4B; P<0.05), but did not affect the expression of ERα mRNA (Fig. 4A). The effect of TNFα in combination with IFNγ on the ERα and ERβ mRNA levels was no different from that of TNFα alone (Fig. 5).

**Discussion**

The present study demonstrated that both ERα and ERβ proteins are expressed in the bovine CL throughout the estrous cycle. Although E has been reported to have both luteotrophic [32, 33] and luteolytic [1, 2, 34] functions in porcine, bovine and rhesus monkey CLs, how E exerts these opposite...
functions remains largely unknown. E action is known to be mediated by ERα and ERβ, which are shifted to a transcriptionally active state following ligand binding. Both ER subtypes regulate gene expression in two ways, either by directly binding to DNA through their estrogen response elements or by interacting with other transcription factors, such as activating protein-1 (AP-1) [35]. ERα and ERβ have been shown to signal in opposite ways when complexed with E from an AP-1 site; the ERα/E complex activated transcription, whereas the ERβ/E complex inhibited transcription [35]. Since ERα expression was higher at the early and mid-luteal stages than at the other stages, ERα may have roles in maintenance of CL function. Moreover, a dramatic decrease in ERα protein expression at the regressed luteal stage could result in progression of structural luteolysis in the bovine CL. In addition, the tissue distributions and relative levels of expression are quite different between ERα and ERβ [7]. Therefore, information about the relative expression levels of ERα and ERβ is needed in order to understand the role of E in tissue co-expressing ERα and ERβ. In the present study, the ratio of ERβ to ERα was significantly higher at the regressed stage than at the other stages. Moreover,
total E binding to ERs (ERα and ERβ) is higher at the regressed luteal stage than at any other stages of the estrous cycle [5]. These findings lead us to hypothesize that E may play important roles via ERβ at the regressed luteal stage in cattle. Indeed, the presence of a growing dominant follicle or injection of E at the late luteal stage has been demonstrated to induce luteolysis in cattle [2]. Collectively, E may act as a luteolytic factor via ERβ at the regressed luteal stage in the bovine CL.

Little is known about the regulation of ERα and ERβ expressions in mammalian tissues. The ERβ protein level in the bovine CL was relatively stable throughout the estrous cycle compared with the ERα protein level, which changed markedly during the luteal phase. Thus, the changes in the ratio of
ERβ to ERα were mainly due to the changes in the ERα protein level. Furthermore, if E exerts different functions via the different receptors, the regulator for ERα seems to be more important for E action in the CL than that for ERβ. In sows, PGF2α administration on Day 17 has been demonstrated to decrease ERα mRNA expression in the CL [20]. In the present study, PGF2α also inhibited ERα mRNA expression in bovine luteal cells. The above findings suggest that PGF2α is one of the regulators of ERα mRNA expression in bovine luteal cells. PGF2α is known to be synthesized and secreted by the endometrium in cattle, with higher levels at the estrus and the regressed luteal stage [17]. Thus, the finding that ERα protein expression was lowest at the regressed luteal stage suggests that luteal ERα protein expression was down-regulated by endometrial PGF2α.

TNFα inhibited ERα mRNA expression in cultured bovine luteal cells in the present study. Down-regulation of ER by TNFα has also been demonstrated in porcine luteal cells [36] and human breast cancer cells [26]. Danforth and Sgagias [26] demonstrated that this process is enhanced in the presence of E. The concentration of E in the bovine ovary is high at the developing luteal (during the first follicular wave) and regressed luteal stages when a dominant preovulatory follicle is present in the ovary [4]. Furthermore, since TNFα is expressed in the bovine CL throughout the estrous cycle [37], cooperative action of TNFα and E may in result in reduction of ERα protein expression at the developing and regressed luteal stages. It is also possible that the reduction in ERα was the indirect effect of TNFα on ERα mRNA expression. TNFα is known to stimulate PGF2α production by bovine luteal cells [37, 38], suggesting that TNFα decreases luteal ERα mRNA expression by stimulating PGF2α production. We expected that IFNγ would enhance the effect of TNFα on ER expression because IFNγ up-regulates TNFα receptors in a variety of cell types [27, 28]. However, TNFα in combination with IFNγ did not alter the ERα and ERβ mRNA expressions compared with treatment with TNFα alone in the present study. It is possible that the incubation time for the luteal cells treated with IFNγ was too short to increase TNFR expression and to decrease significantly the ER mRNA expressions. Otherwise, TNFα could have a stronger effect than IFNγ on ERα mRNA expression. Further studies are needed to clarify this point.

In the present study, PGF2α, TNFα and IFNγ reduced the ERβ mRNA expression in bovine luteal cells. The doses of TNFα (1.45 nM) and IFNγ (1.25 nM) used in the present study did not affect cell viability (data not shown), although we have previously demonstrated that higher concentrations of TNFα and IFNγ have cytotoxic effects on bovine luteal cells [24]. TNFα and IFNγ are present in the bovine CL throughout the luteal phase [37, 39] and are potent stimulators of luteal PGF2α production [37, 38, 40]. Since PGF2α stimulates progesterone production in the bovine CL in vitro [31], we suggest that TNFα and IFNγ act as luteotrophic agents at the concentration used in the present study. Therefore, PGF2α, TNFα and IFNγ may inhibit ERβ mRNA expression to maintain CL function in the cow.

The ERα protein level was higher at the early and mid-luteal stages than at the other stages. The ERβ protein level increased from the early to developing luteal stage, remained at the same level at the mid-luteal stage and decreased thereafter. However, these changes in the ERα and ERβ protein expressions throughout the estrous cycle were not consistent with the changes in ER mRNA expressions reported previously [7]. Moreover, although significant effects of PGF2α, TNFα and IFNγ on ERs were observed at the mRNA level in the present study, Western blot analysis revealed that the levels of ERα and ERβ protein were not affected by any of the above treatments. The discrepancy between the expressions of the ER mRNAs and the expressions of the ER proteins might be due to post-translational processing. Further studies are needed to understand the mechanisms regulating ERα and ERβ expressions, especially translational regulation of ERα and ERβ in the bovine CL.

In conclusion, the present study indicates that PGF2α, TNFα and IFNγ regulate the ERα and ERβ mRNA expressions in bovine luteal cells. Moreover, the changes in the ERβ/ERα ratio that occur throughout the luteal phase suggest that ERα is associated with luteal maintenance. Therefore, a dramatic decrease in ERα at the regressed luteal stage could result in progression of structural luteolysis in the bovine CL.
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References

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