NOTE *Physiology*

Possible Role of Insulin-Like Factor 3 in the Bovine Corpus Luteum

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ABSTRACT. Insulin-like factor 3 (INSL3) is a local regulator in mammalian gonads, but little is known of its function in bovine corpus luteum (CL). Here, we show that RXFP2 protein, the receptor of INSL3, was expressed throughout the estrous cycle and significantly high at the early luteal stage compared to the regressed luteal stage. INSL3 stimulated progesterone secretion, but not prostaglandin F2α and viability in cultured luteal cells. Together, these results suggest that INSL3 plays a luteotropic role as a local regulator in the bovine CL.
KEY WORDS: bovine, corpus luteum, estrous cycle, insulin-like factor 3, insulin-like factor 3 receptor.

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The corpus luteum (CL) is a transient organ that forms from the wall of a Graafian follicle following ovulation and secretes progesterone (P4) [1]. In the cow, luteolysis is occurred by the pulsatile release of endometrial prostaglandin (PG) F2α, which initiates a complex cascade of events that finally interrupt steroidogenesis and induces structural regression of the CL [13].

The insulin-like factor 3 (INSL3) is expressed in pre- and postnatal Leydig cells of the testis and in postnatal theca cells of the ovary [2]. In some mammalian species, INSL3 has been shown to induce testicular descent [16, 26]. In female INSL3 knockout mice, the ovaries had fewer growing follicles, fewer ovulations and fewer resulting corpora lutea, implying a higher rate of follicular atresia [24]. Furthermore, less INSL3 expression is suggested to be associated with atresia of follicles in the cow [9]. Thus, the apparent function of INSL3 seems to rescue follicles and increase the number of ovulations. There are some reports showing that *INSL3* mRNA is expressed strongly in the follicular theca cells and luteal cells in cow [4, 9], and increases from the mid to the late luteal stages in the bovine CL [5]. We also confirmed the similar results, i.e. *INSL3* mRNA is high at the mid luteal stage in bovine CL (data not shown). Although the above findings suggest that INSL3 plays some roles as a local regulator in bovine CL, the roles of INSL3 in the bovine CL remain unclear.

RXFP2 is a specific INSL3 receptor. The INSL3/RXFP2 system has been reported to be involved in several cell functions including differentiation and maturation by acting MAPK cascade in human osteoblasts [7]. Thus, RXFP2 expression is essential for INSL3 to affect the cells in a target tissue. Although *RXFP2* mRNA is expressed in reproductive organs, such as testis and uterus in several species [3, 8, 11, 12, 21], the RXFP2 expression in the bovine CL throughout the estrous cycle is unknown.

In the present study, to clarify the local function of INSL3 in bovine CL, we characterized the patterns of RXFP2 protein expression throughout the luteal phase, and investigated the effects of INSL3 on 1) P4 and PGF2α production by cultured bovine luteal cells and 2) the viability of these cells.

Ovaries were collected from Holstein cows at a local slaughter-house within 10–20 min after exsanguinations. The stage of the estrous cycle was defined as described previously [15, 17]. Ovaries with CLs were classified into the early (Days 2–3 after ovulation), developing (Days 5–6), mid (Days 8–12), late (Days 15–17) and regressed (Days 19–21) luteal stages. After determination of these stages, CL tissues were immediately separated from the ovaries, frozen rapidly in liquid nitrogen and then stored at −80°C until processed for studies of protein expression. For cell culture, ovaries with CLs were submerged in ice-cold physiological saline and transported to the laboratory.

To detect RXFP2 protein by western blotting, CL tissues were homogenized on ice in homogenization buffer by a tissue homogenizer (NS-50; Physcotron; NITI-ON Inc., Chiba, Japan) and then filtered with a metal wire mesh (150 μm). For RXFP2 protein, cytoplasm was isolated from the tissue homogenates by centrifugation at 600 × g for 30 min. The supernatants were then centrifuged at 9,000 × g for 30 min, and the resulting supernatants were used. The protein concentrations of the lysates were determined by the method of Osnes *et al.* [20] using BSA as a standard. Rat testicular tissue was used as a positive control. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [31607–94; Nacalai Tesque, Tokyo, Japan], 10% glycerol, 1% β-mercaptoethanol [137–06862; Wako Pure Chemical Industries, Ltd., Osaka, Japan], pH 6.8) and heated at 95°C for 10 min. Samples (5 μg protein) were subjected

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to electrophoresis on a 7.5% SDS-PAGE for 1 hr at 200 V. The separated proteins were electrophoretically transblotted to a 0.45-μm nitrocellulose membrane (RPN78D; GE Healthcare, Piscataway, NJ, U.S.A.) at 250 mA for 3 hr in transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]), incubated in blocking buffer (5% nonfat dry milk in TBS-T) overnight at 4°C, incubated in TBS-T with primary antibody specific to each protein: RXFP2 antibody (sc-50327; Santa Cruz Biotechnology, Heidelberg, Germany; 1:1,000 diluted in TBS-T, 1 hr at 4°C) and β-actin antibody (A2228; Sigma-Aldrich, Co., St. Louis, MO, U.S.A.; 1:4,000 diluted in TBS-T, 1 hr at 4°C), washed for 10 min in blocking buffer, washed 2 times for 10 min in TBS at room temperature, incubated in TBS-T with a secondary antibody, HRP-linked anti-rabbit-Ig-donkey (NA934; Amersham Biosciences Corp., Piscataway, NJ, U.S.A.; 1:20,000 diluted in TBS-T) for RXFP2 and HRP-linked anti-mouse-Ig-sheep (NA931; Amersham Biosciences Corp., 1:40,000 diluted in TBS-T) for 1 hr and then washed 3 times in TBS-T for 10 min at room temperature. The signal was detected using an ECL Plus Western Blotting Detection System (RPN2132; GE Healthcare). β-actin protein expression was used as an internal control. The intensity of the immunological reaction in the membranes was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health).

Only those CLs classified in the mid luteal stage were collected for cell culture. Luteal tissue was enzymatically dissociated, and luteal cells were cultured as described previously [19]. Dissociated luteal cells from CLs were pooled. The luteal cells were suspended in a culture medium, Dulbecco modified Eagle medium (DMEM) and Ham F-12 medium (D/F; 1:1 [vol/vol]; D8900; Sigma-Aldrich) containing 5% calf serum (16170–078; Life Technologies, Grand Island, NY, U.S.A.) and 20 μg/m*l* gentamicin (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, 10% endothelial cells or fibrocytes and no erythrocytes.

To investigate the effect of INSL3 on P4 and PGF2α production, dispersed luteal cells obtained from two to three CL were seeded at 1.0×10^5 viable cells in 0.5 m*l*, in 48-well cluster dishes (677180; Greiner Bio-One, Frichenhousen, Germany) and cultured in a humidified atmosphere of 5% CO_2 in air at 37.5°C. After 24 hr of culture, culture media were replaced by phenol red-free, serum-free fresh medium, D/F (D2906: Sigma-Aldrich) containing 0.1% BSA, 5 *n*g/m*l* sodium selenite, 5 μg/m*l* transferrin and 20 μg/m*l* gentamicin. The luteal cells were treated with INSL3 (0.1–100 nM, 035–27; Phoenix Pharmaceuticals, Burlingame, AZ, U.S.A.) for 6 hr. Luteinizing hormone (LH: 10 *n*g/m*l*, USDA-bLH B6) was used as a positive control for P4 production. Only CL cells stimulated by LH were used for an experiment. All treatments were conducted in triplicate. The conditioned media from the last 6 hr of culture were collected and stored at −30°C until assayed for P4 and PGF2α.

The concentrations of P4 and PGF2α were determined directly from the cell culture media with an enzyme immunoassay as described previously [23]. The P4 standard curve ranged from 0.391 to 100 *n*g/m*l*, and the effective dose for 50% inhibition (ED_{50}) of the assay was 2.6 *n*g/m*l*. The intra- and inter-assay coefficients of variation were 5.2% and 15.6%, respectively. The PGF2α standard curve ranged from 15.6 to 4,000 *p*g/m*l*, and the ED_{50} of the assay was 330 *p*g/m*l*. The intra- and inter-assay coefficients of variation were 3.8% and 8.2%, respectively.

To investigate the effect of INSL3 on the viability in luteal cells, dispersed luteal cells (2.0×10^5/m*l*) were cultured in 100 μl of D/F medium containing 5% calf serum in 96-well culture dishes (3860–096; Iwaki, Chiba, Japan). After 24 hr of culture, the medium was replaced with D/F medium without phenol red containing 0.1% BSA, 5 *n*g/m*l* sodium selenite (S5261; Sigma-Aldrich) and 5 μg/m*l* holo-transferrin (T3400; Sigma-Aldrich). The cells were then exposed to INSL3 (0.1–100 nM) in the presence or absence of TNF (2.9 nM; kindly donated by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and IFNG (2.5 nM; kindly donated by Dr. S. Inumaru, NIAH, Ibaraki, Japan) for 24 hr. After 24 hr of culture, the medium was replaced with the D/F medium without phenol red. The cells were then exposed to INSL3 with or without TNF and IFNG for 24 hr. After the final 24 hr of culture, the viability of the cells was determined by Dojindo Cell Counting Kit including WST-1 (345–06463; Dojindo, Kumamoto, Japan) as described previously [25]. Briefly, WST-1, a kind of MTT [3- (4,5-dimethyl-2 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], is a yellow tetrazolium salt that is reduced to formazan by live cells containing active mitochondria. The culture medium was replaced with 100 μl D/F medium without phenol red, and a 10 μl aliquot (0.3% WST-1 and 0.2 mM 1- methoxy PMS in PBS, pH 7.4) was added to each well. The cells were then incubated for 4 hr at 38°C. The absorbance was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.; Model 450). In this assay, data were expressed as percentages of the appropriate control values.

All experimental data are shown as the mean ± SEM. The statistical significance of differences in the expression of protein and the concentrations of P4 and PGF2α in culture media and the viability of CL cells were assessed by analysis of variance (ANOVA) with Tukey's post test. In all analyses, a value of $P<0.05$ was considered significant.

As shown in Fig. 1, the RXFP2 protein expression was much higher at the early luteal stage than at the regressed luteal stage, suggesting that INSL3 has a more important role in the CL at the early luteal stage than at other stages.

Bovine luteal cells secrete both P4 and PGF2α [14, 22]. Although PGF2α secreted by the uterus is the main luteolytic factor in cattle, luteal PGF2α [6], like luteal P4 [18], can also act as an anti-luteolytic factor. To determine whether INSL3 controls CL function by regulating P4 and PGF2α secretion in luteal cells, we investigated the effects of INSL3 on P4 and PGF2α production in the bovine luteal cells. INSL3 (≥1 nM) stimulated P4 production in cells cultured for 6 hr (Fig. 2A), whereas INSL3 did not affect PGF2α production in lu-

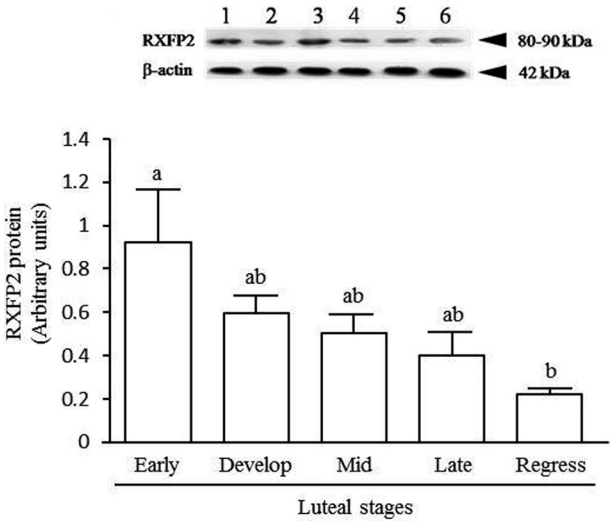

Fig. 1. Changes in the relative levels of RXFP2 protein in bovine CL throughout the luteal phase. Representative western blot samples for RXFP2 and β-actin are shown in the upper panel (lane 1, positive control; lane 2, early; lane 3, developing; lane 4, mid; lane 5, late; lane 6, regressed luteal stages). The blot was incubated with primary antibodies against RXFP2, β-actin and was then incubated with secondary antibody conjugated to horseradish peroxidase. The resultant signal was detected by chemiluminescence and was quantitated by computer-assisted densitometry. The RXFP2 protein levels are expressed relative to the amount of β-actin protein (n=4). All values are the mean ± SEM of the densitometric analysis of RXFP2 protein levels in the CLs. Different letters indicate significant differences (P<0.05), as determined by ANOVA with Tukey's post test.

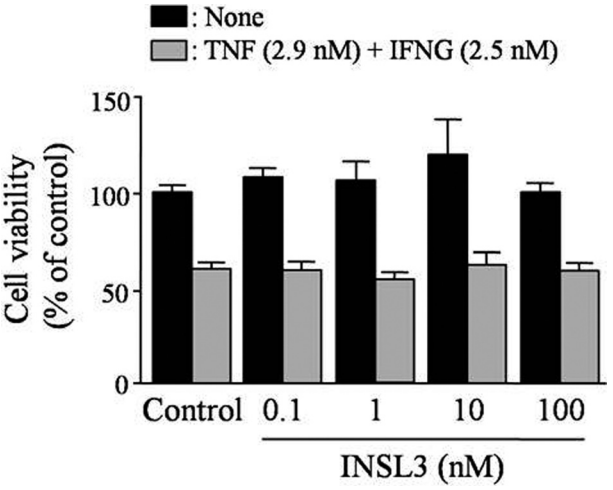

Fig. 3. Effect of INSL3 on bovine luteal cell viability. The cells were treated with INSL3 (0.1-100 nM) in the presence or absence of cytokines (TNF: 2.9 nM, IFNG: 2.5 nM) for 24 hr, followed by treatment with INSL3 (0.1-100 nM) in the presence or absence of TNF and IFNG for 24 hr. After the final 24 hr of culture, the cell number was determined by optical density at 450 nm in a WST-1 assay. Experiments with isolated cells were performed three times each with separate cell preparations. Data are the mean ± SEM of three separate experiments. All values are expressed as a percentage of mean of cytotoxicity in untreated control (defined in Materials and Methods), as determined by ANOVA with Tukey's post test.

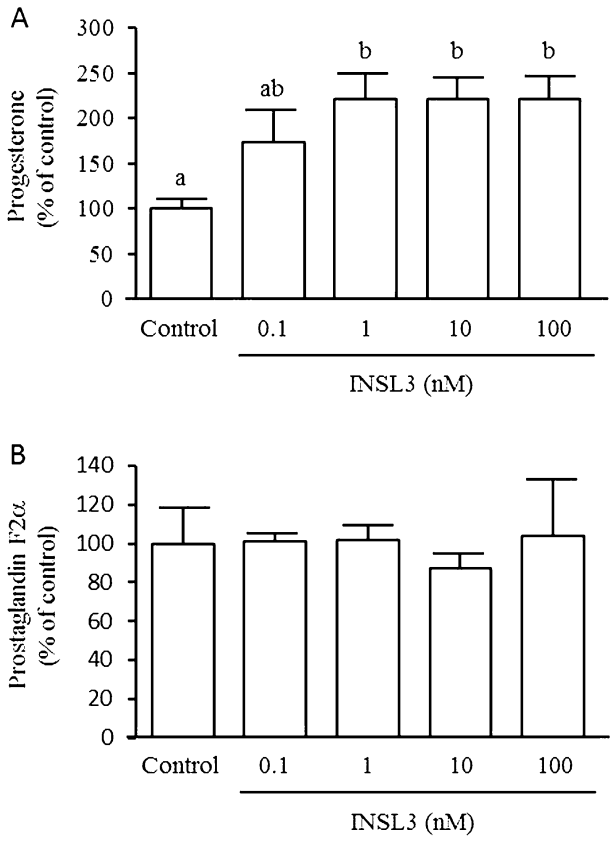

Fig. 2. Effect of INSL3 on progesterone (A) and prostaglandin F2α (B) production by cultured bovine luteal cells from 3-4 separate experiments. The cells were exposed to INSL3 (0.1, 1, 10 and 100 nM) for 6 hr. All values are expressed as a percentage of the mean of control value. The concentrations of P4 and PGF2α in the control were 127.5 ± 33.0 *n*g/m*l* and 48.1 ± 3.5 *p*g/m*l*, respectively. Different letters indicate significant differences (P<0.05), as determined by ANOVA with Tukey's post test.

teal cells (Fig. 2B), suggesting that INSL3 helps to maintain CL function by stimulating P4 secretion. This is consistent with the finding that RXFP2 protein expression was much higher at the early and developing luteal stages when blood P4 concentration increases conspicuously [10].

We have previously suggested that P4 affects the maintenance of CL by suppressing the apoptosis of bovine luteal cells in a culture system [18]. Moreover, apoptosis of luteal cells was greater in INSL3-deficient mice than in wild-type mice [24]. However, a single treatment of INSL3 did not affect the viability of cultured bovine luteal cells (Fig. 3). The discrepancy might be due to differences between species and/or the experimental models used (*in vitro* vs. *in vivo*) [24]. We previously induced apoptosis in cultured bovine luteal cells by treating the cells with TNF and IFNG [25]. In the present study, the treatment with INSL3 did not affect the cell death induced by TNF and IFNG (Fig. 3), suggesting that INSL3 does not play a role in promoting the structural regression of bovine CL.

The overall findings suggest that INSL3 acts as a local

promoter of bovine luteal function by increasing P4 production at the early and developing luteal stages.

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