Interleukin-6 up-regulates the expression of rat luteinizing hormone receptors during granulosa cell differentiation

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Interleukin-6 (IL-6) is produced in granulosa cells under normal physiological conditions, including during ovulation. However, the roles of IL-6 in ovarian function, including regulation of LH receptor (LHR) expression in granulosa cells, have not been explored in detail. The aim of this study was to identify the mechanism underlying the effect of IL-6 on LHR expression in the granulosa cells of female Wistar rats. Our results indicated that IL-6 clearly enhanced the FSH-induced LHR mRNA expression in a dose-dependent manner and did not stimulate cAMP accumulation by itself. The membrane protein level of LHR, assessed by a binding assay, was increased by FSH and was further enhanced by association with IL-6. Results of the luciferase assay, using promoter constructs of LHR 281 bp upstream of the translational start site, revealed that IL-6 increased promoter activity induced by FSH, but this effect was not observed with treatment by IL-6 alone. This ability of IL-6 to enhance FSH-induced LHR mRNA expression was blocked by the JAK pathway inhibitor, but not by the ERK1/2 inhibitor. Thus, we speculated that this IL-6 activity might be mediated by the JAK/STAT pathway. Additionally, IL-6 augmented FSH-induced IL-6Rα mRNA expression and FSH elevated IL-6 production in granulosa cells, which indicates that IL-6 may positively regulate paracrine and autocrine actions in granulosa cells. These results suggest that IL-6 up-regulates FSH-induced LHR production by increasing mRNA transcription, and JAK/STAT3 signaling is required for up-regulation by IL-6 in granulosa cells.

Pituitary gonadotropins are key hormones in the regulation of ovarian follicles. Both LH and FSH act through stimulatory G protein-coupled receptors and primarily transduce their signals through activation of adenylyl cyclase and the production of cAMP (1). The expression of LHR is one of the major markers of FSH-induced differentiation of granulosa cells. Primary cultures of rat granulosa cells obtained from immature female rats pretreated with estradiol are a frequently used model for studying granulosa cell differentiation. Exposure to FSH or compounds that increase the levels of intracellular cAMP results in a dose-dependent increase in the expression of LHR in cultured granulosa cells (2). In addition to FSH, many local factors, including cytokines, are involved in the regulation of LHR expression (3, 4). Since LHR play a key role in follicular maturation, ovulation and steroidogenesis (5–7), elucidating the effect of local factors on LHR expression is important for understanding the reproductive mechanisms in the ovary.

Interleukin-6 is a cytokine originally identified as a B cell differentiation factor (8) and is a representative cytokine producing pleiotropic effects on various cell types (9). IL-6 binds with a transmembrane receptor, IL-6 receptor α (IL-6Rα), or soluble IL-6R (sIL-6R), and binding of IL-6 to IL-6Rα induces heterodimerization with its coreceptor gp130 (10). Although gp130 is expressed ubiquitously in many organs (11), IL-6Rα is mainly expressed in hepatocytes and leukocytes (12), as well as in granulosa cells (13, 14). The dimerized gp130 triggers activation of Janus Kinases (JAKs) which in turn phosphorylate the signal trans-

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Abbreviations:

ducer and activator of transcription-3 (STAT3) (10). Engagement of gp130 also recruits Src homology domain-containing protein tyrosine phosphatase –2(SHP-2), leading to activation of the ERK/MAPK cascade (10).

Under normal physiological conditions (eg, during ovulation), IL-6 is produced in granulosa cells (15), but studies have suggested that IL-6 negatively affects reproductive functions in the ovary. For example, several reports have shown that IL-6 suppresses FSH-induced estradiol and progesterone in granulosa cells (16–18). The inhibitory effects of IL-6 on gonadotropin receptor have also been described (19, 20). Conversely, IL-6 also performs important physiological functions in the ovary, acting as a potent autocrine regulator of ovarian cumulus cell function, COC expansion, and oocyte competence (21). Therefore, the roles of IL-6 in ovarian function remain to be elucidated.

In the present study, we examined the effect of IL-6 on FSH-induced LH-R mRNA and protein expression levels and mechanisms of regulation in rat primary granulosa cells. Our results indicated that IL-6 increased FSH-induced expression of LHR, which is mediated by the JAK/STAT pathway.

Materials and Methods

Hormones and reagents

Recombinant human FSH was supplied by Dr. A. Parlow and the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health [NIH]). DMEM/Ham's nutrient mixture F-12, diethylstilbestrol (DES), and 8-bromo-cAMP (8-Br-cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human IL-6 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). U0126 and Janus kinase (JAK) inhibitor I were obtained from Calbiochem (La Jolla, CA, USA). Gentamicin sulfate and fungizone were purchased from Invitrogen Corp. (Carlsbad, CA, USA). The RNA labeling kit and nucleic acid detection kit were purchased from Roche Diagnostics (Indianapolis, IN, USA).

Animals

Immature female Wistar rats (Japan SLC, Inc.) were maintained at all times according to the NIH guidelines for the care and use of laboratory animals and the policies of the Gunma University Animal Care and Use Committee. Animals were housed in a temperature-and light-controlled room (12 hours light, 12 hours dark cycle; lights on at 0600) with food and water provided ad libitum.

Granulosa cell cultures

Granulosa cells were obtained from immature female Wistar rats injected daily for 4 days with 2 mg DES in 0.2 mL of sesame oil. The ovaries were then excised, and the granulosa cells were released by puncturing the follicles with 26-gauge needles. Granulosa cells were washed and collected by brief centrifugation, and the cell viability was determined by trypan blue exclusion. The granulosa cells were then cultured in DMEM/Ham's nutrient mixture F-12 supplemented with 20 mg/L gentamicin sulfate, 500 μg/L fungizone, and 1 g/L BSA on collagen-coated plates in a humidified atmosphere under 5% CO_2 at 37°C.

RNA isolation and northern blot analysis

Granulosa cells were cultured in 60-mm dishes containing 5×10^6 viable cells in 5 mL of medium, and the reagents were added to the medium after 24 hours of cell culture. The granulosa cells were further incubated, and the cultures were stopped at the selected times as indicated by using total RNA extraction reagent Isogen (Nippon Gene, Toyama, Japan). The final RNA pellet was dissolved in diethylpyrocarbonate-treated H_2O. Total RNA was quantified by measuring the absorbance of samples at 260 nm. For the northern blot analysis, 15 μg of total RNA from each dish were separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Biodyne; Pall Corp., Pensacola, FL). Rat LHR cDNA was prepared as described previously and linearized with *Bgl*II (22). Digoxigenin-labeled LHR cRNA probes corresponding to bases 440–2560 were produced by in vitro transcription with T3 RNA polymerase and an RNA labeling kit (Roche Diagnostics). A digoxigenin-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was obtained by the same method. In accordance with the standard protocol for the nucleic acid detection kit (Roche Diagnostics, Tokyo, Japan), the membranes were then exposed on Kodak XAR film (Eastman Kodak Co., Rochester, NY).

Reverse transcription and quantitative RT-PCR

Isolated RNAs (2 μg of each sample) from the granulosa cell cultures were treated with DNaseI (Invitrogen) to eliminate residual genomic DNA. These RNAs were reverse transcribed with random primers, 10 mM deoxynucleoside triphosphate mix, and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The reactions were incubated for 5 minutes at 25°C, 60 minutes at 50°C, and 15 minutes at 70°C in a thermal cycler. To remove complementary RNA (cRNA), RNaseH was added to the cDNAs and incubated for 20 minutes at 37°C. Real-time RT-PCR was performed using TaqMan gene Expression assays (luteinizing hormone (LH)/choriogonadotropin receptor: Rn00564309_m1, Eukaryotic 18s rRNA: Hs99999901_s1 as an internal control; Applied Biosystems), completed according to manufacturer instructions. Relative quantifications of mRNA were carried out using the comparative threshold (CT) cycle method. For each sample, we normalized the gene CT value using the formula $\Delta CT = CT$ gene-CT 18S. For relative expression levels, the following formula was used: $\Delta\Delta CT = \Delta CT$ sample-ΔCT calibrator. This value was then used to plot gene expression employing the formula $2^{-\Delta\Delta CT}$. The interleukin-6 receptor (Rn00566707_m1) expression level was measured in the same way as in the LHR mRNA assays.

Receptor binding assay

Granulosa cells were cultured in 24-well plates containing 5×10^5 viable cells in 0.5 mL of medium. After 24 hours incubation, reagents were added to the medium for 72 hours. Thereafter, the cells were placed on ice and quickly washed three times

with 0.4 mL cold medium. Then, the granulosa cells were incubated in a 1:1 (vol/vol) mixture of DMEM-Ham's F-12 medium containing 0.1% BSA (pH 7.4) at 37°C with 1.5×10^5 cpm [^{125}I]hCG (5 ng, 30,000 cpm/ng). The hCG was iodinated according to the chloramine-T method (23). The incubation medium was removed after 1 hour of incubation, and the cells were washed twice with 0.4 mL of medium. The amount of radioactivity remaining in the well (cell-bound hormone) was then quantified by β-spectrometry. Nonspecific binding was determined by adding excess unlabeled hCG (50 IU/well).

Luciferase reporter assay

Plasmid pGL3-basic is a luciferase vector lacking the eukaryotic promoter and enhancer sequences (Promega Corp., Madison, WI). The PGL3-control contains a simian virus 40 promoter and a simian virus 40 enhancer inserted into the structure of pGL3-basic (Promega). The fragment of the 5′-flanking region from bp –281 to bp –1 (relative to the translational initiation site), which included the transcriptional regulating region of the rat LHR, was kindly provided by Dr. Kaoru Miyamoto (Department of Biochemistry, University of Fukui, Fukui, Japan) generated from the gDNA as described previously (24). To evaluate promoter activity, these fragments were ligated to a luciferase reporter vector (pGL3-basic) and designated LH-R(281)-Luc. Using FuGENE 6 reagent (Promega), we transfected 300 ng of plasmid DNA into the primary rat granulosa cell culture plates (1.5×10^5 cells per 0.5 mL of medium in a 24-well culture plate). To assay the regulatory elements, the granulosa cells were cultured for 24 hours in a hormone-free medium before transfection. Twenty-four hours after transfection, the cells were treated with hormones for 3 hours. The cells were then harvested, and the luciferase activity was measured. The luciferase assay was performed using the dual-luciferase reporter system (Promega), in which the transfection efficiency was monitored by cotransfected pRL-CMV-Rluc, an expression vector of Renilla luciferase.

SDS-PAGE and western blot analysis

Granulosa cells were cultured in 60-mm dishes containing 5×10^6 viable cells in 5 mL of medium. Twenty-four hours later, the cells were incubated with or without inhibitors for 1 hour at 37°C, and then stimulated with FSH plus IL-6. The cells were lysed with RIPA buffer (pH 7.4, 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease complete inhibitor). The lysate (20 μg) was then resolved on 7.5%–12% sodium dodecyl sulfate gels and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, expressions of the different proteins were determined overnight at 4°C with the following antibodies: rabbit anti-phospho-p44/42MAPK (ERK1/2) (Cell Signaling Technology; Beverly, MA, USA; no. 9101, 1:5000), rabbit anti-p44/42 MAPK(ERK1/2) (Cell Signaling Technology; no. 9102, 1:2500), rabbit anti-phospho-STAT3 (Cell Signaling Technology; no. 9131, 1:2500), and rabbit anti-STAT3 (Cell Signaling Technology; no. 9132, 1:2500). After washing in TBS/T, the membranes were incubated at room temperature for 60 minutes with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G (IgG) antibody (Bio-Rad Laboratories, Hercules, CA, USA; no. 170–6515, 1:40000). The proteins were finally visualized using enhanced chemiluminescence (Immobilon Western; Millipore, Billerica, MA, USA). Luminescence was quantified by scanning the films with a CCD camera and analyzing the digitized data with the NIH Image 1.60 computer program.

Data Analysis

Data represented the means ± SEM from at least three independent experiments. Comparisons between groups were performed by one-way ANOVA. The significance of the differences between the mean values of the control group and each treated group was determined using the Scheffe multiple-comparison test. A value of $P < .05$ was considered significant.

Results

IL-6 enhanced the activity of FSH on expression of LHR

To investigate the effect of FSH and IL-6 on LHR mRNA expression, we analyzed the LHR mRNA in the primary rat granulosa cell culture. Granulosa cells were cultured for 24 hours after plating on a cell culture dish, and then the cells were cultured in the absence or presence of FSH(30 ng/mL) with or without increasing concentrations of IL-6 (0.1–10 ng/mL) for 72 hours (Figure 1A). Levels of basal LHR mRNA were negligible and remained unchanged by treatment with IL-6 alone. In contrast, treatment with FSH produced an expected substantial increase in LHR mRNA levels, and concurrent treatment with increasing concentrations of IL-6 resulted in dose-dependent increases in FSH-induced LHR mRNA.

Granulosa cells were also cultured for 24–96 hours in the presence of FSH (30 ng/mL) with or without IL-6 at a concentration of 10 ng/mL (Figures 1B, C). Basal LHR mRNA levels remained low throughout the 96 hours incubation period and the levels were not significantly affected by treatment with IL-6 alone (data not shown). The concurrent treatment with IL-6 resulted in significant augmentation of the FSH-induced LHR mRNA from 48 hours until 96 hours.

It is known that the action of FSH is mediated by cAMP and that a considerable amount of cAMP accumulates in granulosa cells following FSH. It has also been shown that FSH increases intracellular cAMP levels (25, 26); however, IL-6, either alone or in combination with FSH, had no effect on the intracellular cAMP levels (Supplemental Figure 1). We therefore examined the effect of IL-6 on the expression of 8-Br-cAMP-induced LHR mRNA (Figure 1D). Treatment with 8-Br-cAMP (1–2 mM) plus IL-6 (10 ng/mL) for 72 hours produced a significant increase in LHR mRNA, suggesting that IL-6 activated the action of FSH at sites distal to cAMP generation in granulosa cells.

To measure the effect of IL-6 on membrane LHR pro-

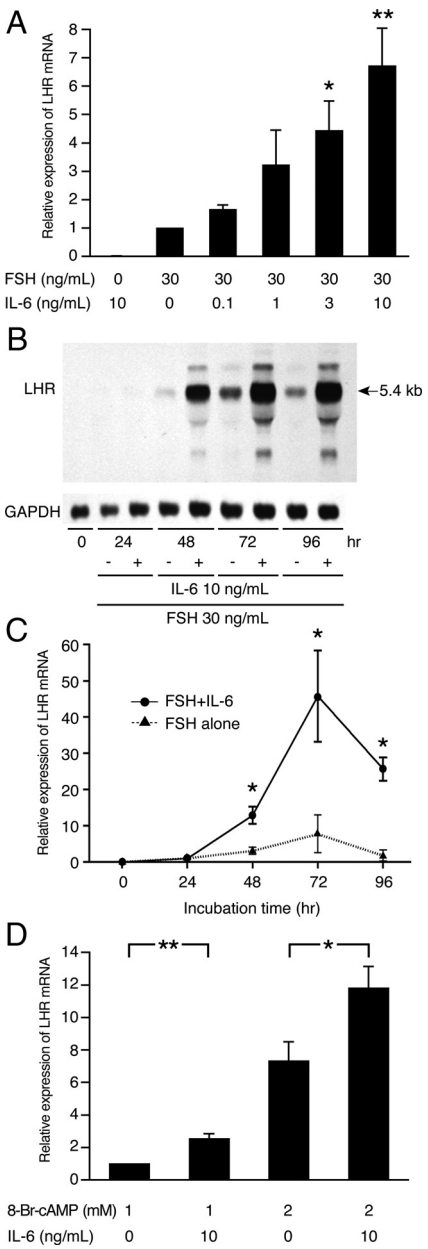

Figure 1. Effects of IL-6 on LHR mRNA expression in rat granulosa cells. (A) Granulosa cells from DES-primed immature rats were cultured for 24 hours. These cells were then further incubated with FSH (30 ng/mL) alone or with FSH plus increasing concentration of IL-6 for 72 hours. Levels of LHR mRNA were measured by quantitative RT-PCR using TaqMan primers. The amount of LHR mRNA with FSH alone was taken as a ratio of 1. Data in the bar graphs represent the means ± SEM of four independent experiments. * indicates increase from the control (FSH alone) value, $P < .05$; ** indicates increase from the control value, $P < .01$. (B, C) Granulosa cells from DES-primed immature rats were cultured for 24 hours. These cells were then further incubated with FSH (30 ng/mL) alone or with FSH plus IL-6 (10 ng/mL) for 24–96 hours. The northern blot is representative of three experiments. Levels of LHR mRNA were measured by quantitative RT-PCR using TaqMan primers. The amount of LHR mRNA in FSH alone at 24 hours was taken as a ratio of 1. Data in the bar graphs represent the means ± SEM of three independent experiments. * indicates increase from the control (FSH alone) value of the respective phase, $P < .05$. (D) Granulosa cells from DES-primed immature rats, cultured for 24 hours, then incubated with 8-Br-cAMP (1 or 2 mM) alone or with 8-Br-cAMP plus IL-6 (10 ng/mL) for 72 hours. Levels of LHR mRNA were measured by quantitative RT-PCR using TaqMan primers. The amount of LHR mRNA with 8-Br-cAMP alone (1 mM) was taken as a ratio of 1. Data in the bar graphs represent the means ± SEM of three independent experiments. ** indicates increase from the 8-Br-cAMP alone (1 mM) value, $P < .01$. * indicates increase from the 8-Br-cAMP alone (2 mM) value, $P < .05$.

tein levels, ^{125}I-hCG binding assay was performed (Figure 2). Granulosa cells were cultured in the absence or presence of FSH (30 ng/mL) with or without IL-6 (10 ng/mL) for 72 hours. Basal-specific LH binding was negligible and remained unchanged in response to treatment with IL-6 alone, whereas treatment with FSH produced, as expected, a substantial increase in specific LH binding, and concurrent treatment with IL-6 produced greater change compared with that produced by FSH alone.

IL-6 enhances the activity of FSH on IL-6Rα mRNA expression

IL-6Rα has also been detected on the surface of granulosa cells (13, 14). In order to examine the possible effect of FSH and IL-6 on IL-6Rα mRNA expression, granulosa cells were cultured for 24–96 hours in the presence of FSH (30 ng/mL) with or without IL-6 at a concentration of 10 ng/mL (Figure 3). FSH increased IL-6Rα mRNA, and concurrent treatment with IL-6 resulted in significant augmentation of the FSH-induced IL-6Rα mRNA from 24 hours until 48 hours.

IL-6 enhances FSH-induced activity of the LHR promoter

To understand the mechanism through which FSH and IL-6 stimulation induce LHR expression, we next investigated the effect of LHR promoter activity using IL-6. In

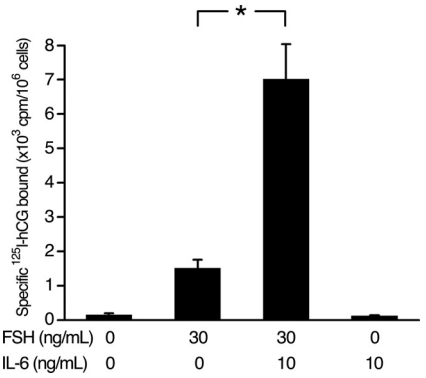

Figure 2. Effect of IL-6 on FSH-induced LHR in rat granulosa cells. Granulosa cells from DES-primed immature rats were cultured for 24 hours. These cells were then further incubated with FSH (30 ng/mL) alone or with FSH plus IL-6 (10 ng/mL). After 72 hours, the levels of LHR were determined by ^{125}I-hCG binding assays. Data in the bar graphs represent the means ± SEM of three independent experiments. * indicates increase from the FSH alone value, $P < .01$.

a previous experiment, Yoshino et al (24) reported that the LHR 5′-flanking region between –171 and –137 bp is essential for basal expression of LHR gene. Therefore, we selected the proximal 281 bp of the LHR 5′-flanking region for this experiment. Granulosa cells transiently transfected with luciferase-reporting plasmids [LH-R(281)-Luc] responded to FSH (30 ng/mL) with significantly enhanced activity. Moreover, treatment with IL-6 (10 ng/mL) enhanced the FSH-induced activity of the LHR promoter. However, treatment with IL-6 alone did not influence the activity of the LHR promoter (Figure 4). Transcription stability analysis using actinomycin-D was then performed to assess the rate of degeneration of LHR mRNA transcripts, but no significant differences were observed between the half-lives of LHR mRNA from FSH alone and FSH plus IL-6 (Supplemental Figure 2).

FSH combined with IL-6 induced phosphorylation of ERK1/2 and STAT3, and inhibitors of these signals selectively suppressed each of these pathways

It was previously reported that IL-6 can activate both the ERK1/2 and STAT3 pathways (10), while ERK1/2 can be stimulated by FSH alone (27). To confirm these responses in rat granulosa cells, time course studies of western blot were performed. Western blot analysis revealed that phosphorylation of ERK1/2 was maximal after 15 minutes exposure of IL-6, FSH, and FSH + IL-6. On the other hand, phosphorylation of STAT3 was also maximal after 15 minutes exposure of IL-6 and FSH + IL-6, whereas FSH did not activate the STAT3 pathway (Figures 5A, B, C, D, E, and F). At this same time point, we assessed the selectivity of two inhibitors (U0126; inhibitor of the upstream ERK-activating kinase MEK, and JAK Inhibitor I: inhibitor of JAK1/2/3) by measuring ERK1/2 and STAT3 phosphorylation. Granulosa cells were pretreated for 1 hour with U0126 (1 μM) or JAK inhibitor I (0.1 μM), and the phosphorylation of ERK1/2 or STAT3 was then measured at the end of a 15-minute incubation period with FSH (30 ng/mL) plus IL-6 (10 ng/mL). From Figures 5G and 5I (ERK1/2), and Figures 5H and 5J (STAT3), it can be seen that U0126 significantly inhibited ERK1/2 phosphorylation without affecting STAT3 phosphorylation, whereas JAK Inhibitor I inhibited STAT3 phosphorylation without affecting ERK1/2 phosphorylation.

The effect of IL-6 on up-regulation of LHR mRNA was mediated by the STAT3 pathway

To identify the signal pathways involved in up-regulation of LHR mRNA by IL-6, we analyzed the expression of LHR mRNA in the absence or presence of U0126 or JAK Inhibitor I. Granulosa cells were pretreated for 1 hour with U0126 (1 μM) or JAK inhibitor I (0.1 μM), and the cells were then cultured for 72 hours in the presence of FSH (30 ng/mL) with IL-6 (10 ng/mL) (Figure 6). JAK Inhibitor I was observed to significantly reduce LHR mRNA expression induced by FSH + IL-6, but U0126 did not. These results demonstrated that JAK/STAT is an important pathway through which IL-6 affects up-regulation of LHR mRNA.

Discussion

In the present study, we have shown for the first time that IL-6 enhanced the expression of FSH-induced LHR and

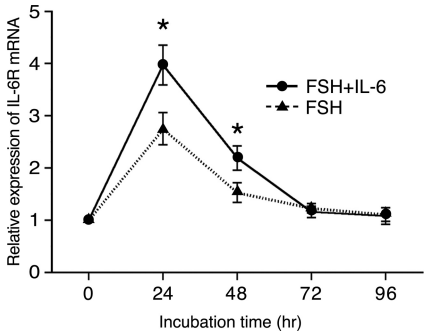

Figure 3. Time course of IL-6 effect on FSH-induced IL-6Rα mRNA. Granulosa cells from DES-primed immature rats were cultured for 24 hours. These cells were then further incubated with FSH (30 ng/mL) alone or with FSH plus IL-6 (10 ng/mL). Levels of IL-6Rα mRNA were measured by quantitative RT-PCR using TaqMan primers. The amount of IL-6Rα mRNA in FSH alone at 0 hours was taken as a ratio of 1. Data in the bar graphs represent the means ± SEM of three independent experiments. * indicates increase from the control (FSH alone) value of the respective phase, $P < .05$.

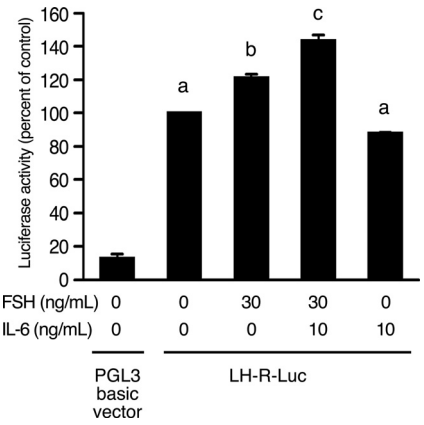

Figure 4. Effects of FSH and IL-6 on LH-R-Luc expression in rat granulosa cells. Granulosa cells from DES-primed immature rats were cultured for 24 hours under hormone-free conditions and then cotransfected with LH-R(281)-Luc and pRL-CMV-Rluc. Twenty-four hours after transfection, cells were treated with FSH (30 ng/mL) alone or with IL-6 (10 ng/mL) for 3 hours, and then cells were lysed and assayed for luciferase activity. Luciferase activities were corrected for the amount of Renilla luciferase activity detected in each lysate. The luciferase activity of the sample treated with no reagent was taken as 100%. Data in the bar graph represents the mean ± SEM of the three independent experiments. Letters a, b and c represent significant differences ($P < .01$) between them.

did not stimulate accumulation of cAMP by itself in immature rat granulosa cells. We have also demonstrated that in granulosa cells, IL-6 up-regulates FSH-induced LHR expression via the JAK/STAT signal transduction pathway.

In the ovary, a variety of cytokines plays important roles in the control of ovarian functions. The most well-known cytokines related to reproduction are interleukins, colony stimulating factors (CSFs), and interferons (IFNs) (28). In our initial investigation of the effect of IL-6 on LHR mRNA expression, the protein level of LHR was assessed by binding assay and was found to increase in response to FSH, and this increase was further enhanced by association with IL-6 (Figure 2). Our data were also consistent with results indicating that changes in the number of cell-surface LH receptors are closely paralleled by changes in LH receptor mRNA levels (29).

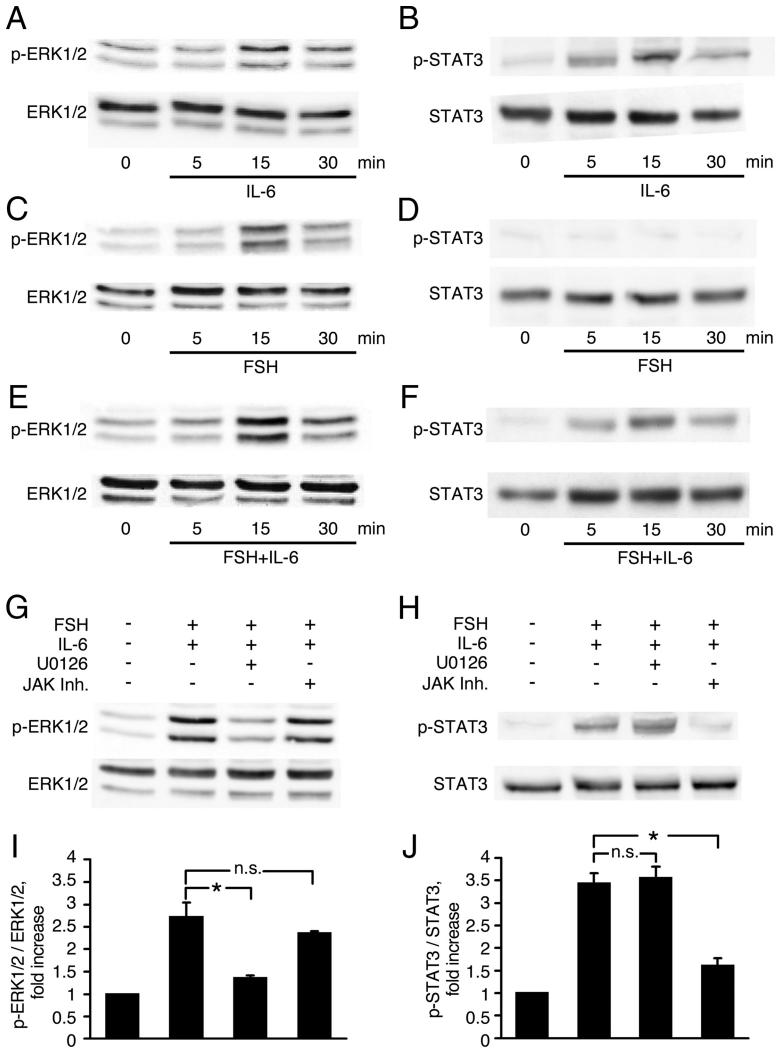

Figure 5. (A, B, C, D, E, F) Effects of IL-6 and/or FSH on phosphorylation of ERK and STAT3 in rat granulosa cells. Granulosa cells from DES-primed immature rats were cultured 24 hours, and then treated for the indicated times with IL-6 (10 ng/mL) and/or FSH (30 ng/mL). Whole cell lysates were used for western blot to detect levels of phosphorylated ERK(p-ERK) (A, C, and E), and phosphorylated STAT3 (p-STAT3) (B, D, and F). The detection of ERK1/2 or STAT3 protein served as a loading control. The blot is representative of three independent experiments. (G, H, I, J) Effects of pathway blockade in FSH plus IL-6-induced phosphorylation of ERK and STAT3 in rat granulosa cells. Granulosa cells from DES-primed immature rats were cultured 24 hours. These cells were pretreated with MEK inhibitor U0126 (1 μM) or JAK1/2/3 inhibitor JAK Inhibitor I (0.1 μM) for 1 hour. Cells were then treated for 15 minutes with FSH (30 ng/mL) plus IL-6 (10 ng/mL). Whole cell lysates were used for western blot to detect levels of phosphorylated ERK(p-ERK) (G), and phosphorylated STAT3 (p-STAT3) (H). The detection of ERK1/2 or STAT3 protein served as a loading control. The blot is representative of three independent experiments. Relative ratios of phosphorylated proteins to total proteins were quantified by scanning the films obtained from western blotting, and digitized data were analyzed with the NIH Image 1.60 software program (I, J). The digitized data of untreated groups were taken as a ratio of 1. The absorbance values in the bar graphs represent the means ± SEM of three independent experiments. * indicates decrease from FSH plus IL-6 value, $P < .01$.

We also investigated whether the regulation of LHR mRNA by FSH and IL-6 was dependent on gene transcription and/or receptor mRNA stability. Yoshino et al (24) reported that the 5′-flanking region of Lhcgr between –171 and –137 bp is essential for basal expression of the Lhcgr gene in rat granulosa cells. It has also been reported that the three Sp-1 binding sites, located at –83,-103,-143 bp of the upstream region and within the proximal portion of the 5′-flanking region, appear to be important for LHR transcription in rat granulosa cells (30). Therefore, we measured luciferase activity using promoter constructs of LHR containing 281 bp upstream of the translational start site. The luciferase assay showed that FSH significantly enhanced the activity of the 281 bp LHR 5′-flanking region, and that treatment with IL-6 alone did not influence the activity of the LHR promoter, but increased promoter activity induced by FSH (Figure 4). On the other hand, IL-6 did not appear to influence FSH-induced LHR mRNA stability (Supplemental Figure 2). These data demonstrated that IL-6 increases promoter activity of LHR induced by FSH, but does not influence LHR mRNA stability.

Under normal physiological condition, such as during ovulation, IL-6 is produced in granulosa cells (15). The receptor for IL-6 is composed of an IL-6α subunit and gp130, and IL-6 binding produces

the gp130 homodimer, which activates Janus kinases (JAKs) and subsequently phosphorylates the signal transducer and activator of transcription-3 (STAT3) (10). Engagement of gp130 also recruits the signal-transducing molecule Src homology domain-containing protein tyrosine phosphatase-2 (SHP-2), which leads to activation of the ERK/MAPK cascade (10). In addition to IL-6, IL-6Rα was also detected on the surface of human granulosa cells (13, 14). As seen in Figure 3, FSH increased IL-6Rα mRNA, while concurrent treatment with IL-6 resulted in significant augmentation of FSH-induced IL-6Rα mRNA expression. It has been reported that FSH elevates IL-6 release from granulosa cells through cAMP-dependent mechanisms (31). Although these data indicate the positive paracrine and/or autocrine regulation of IL-6 action in granulosa cells, it is possible that the immune cells in the ovary contribute to the product of IL-6, which would be consistent with several studies suggesting that IL-6 is produced by immune cells such as T-cells, B-cells, monocytes (10, 12).

Conversely, other studies have suggested that IL-6 may adversely influence ovarian functions. For example, IL-6 reportedly suppressed FSH-induced production of estradiol and progesterone in granulosa cells (16–18). In contrast to our study, the inhibitory action of IL-6 on the expression of FSH induced LHR mRNA has also been reported (20). This difference between result of this previous study and our current study might be attributed to variations in in vivo hormonal treatment prior to primary cell culturing and/or the in vitro culture system, itself. Following diethylstibestrol (DES) treatment in our experiment, granulosa cells expressed FSH receptors but not LH receptors (32, 33), while PMSG treatment resulted in the expression of both LH and FSH receptors in cultured granulosa cells in the absence of hormone treatment (20, 33) .

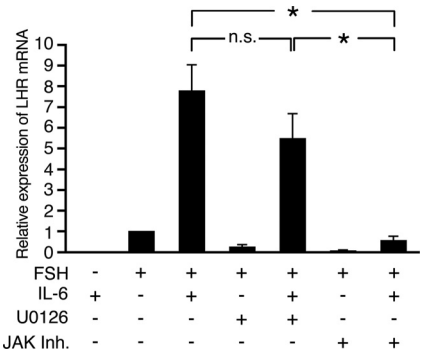

Figure 6. Effects of ERK and JAK/STAT inhibition on LHR mRNA expression in rat granulosa cells. Granulosa cells from DES-primed immature rats were cultured for 24 hours. These cells were pretreated with MEK inhibitor U0126 (1 μM) or JAK1/2/3 inhibitor JAK Inhibitor I (0.1 μM) for 1 hour. Cells were then treated for 72 hours with FSH (30 ng/mL) alone or with FSH plus IL-6 (10 ng/mL). LHR mRNA levels were measured by quantitative RT-PCR using TaqMan primers. The amount of LHR mRNA with FSH alone was taken as a ratio of 1. Data in the bar graphs represent the means $\pm$ SEM of five independent experiments. For each treatment, statistically significant differences in mRNA expression are indicated with asterisks ($*P < .01$).

We confirmed that IL-6 stimulated the phosphorylation of both ERK1/2 and STAT3, while FSH stimulated ERK1/2 alone in rat granulosa cells (Figures 5A, B, C, and D). At the same time, we determined that treatment combining FSH and IL-6 stimulated both ERK1/2 and STAT3 phosphorylation (Figures 5E, and F). We also identified conditions to selectively inhibit ERK1/2 and STAT3 pathways (Figures 5G, H, I, and J). To identify the signal transduction pathway involved in activation of FSH and its effect on LHR expression by IL-6, we investigated the effect of IL-6 on FSH-induced LHR mRNA, using ERK1/2 and JAK inhibitors. The JAK pathway inhibitor blocked the effects of IL-6 on FSH action for LHR mRNA expression, but the ERK1/2 pathway inhibitor produced no significant effect (Figure 6). Therefore, we speculated that IL-6 activity might be mediated by the JAK/STAT pathway.[2

In terms of the effects of FSH on granulosa cell differentiation, cAMP-dependent protein kinase A (PKA) mediates most of the action of FSH (34, 35). In addition, the expression of FSH-stimulated LHR mRNA is dependent on the PKA pathway in granulosa cells (36). The activation of PKA results in the phosphorylation of certain transcription factors that bind with cAMP-responsive element (CRE) to induce or repress the expression of FSH-responsive genes. These cAMP responsive factors are known as cAMP-response element-binding proteins (CREB). Previous studies have reported that, although a classical CRE is not present in the 5′-flanking region of Lhcgr (37), the Lhcgr gene promoter region forms a complex that increases significantly following pretreatment with 8Br-cAMP, and is essential for cAMP responsiveness of the rat Lhcgr gene (30). Therefore, we speculated that IL-6 inducible transcription factor might interact with the factors induced by FSH and subsequently enhance transcriptional activity on the 5′-flanking region of the Lhcgr gene. Our results indicated that IL-6 alone does not change the amount of LHR mRNA, but it is effective when combined with FSH action. These data indicate that the mechanisms of IL-6-induced JAK/STAT phosphorylation, which enhance LHR mRNA expression, do not represent a direct effect, but instead involve activation of a component downstream of the PKA pathway. Additionally, another study indicated that STAT5-independent nuclear JAK2 signaling plays an essential role in adrenal steroidogenesis by increasing stability of the transcriptionally active CREB (38). It is therefore possible that IL-6 may enhance the expression of LHR mRNA through a similar mechanism.

It has also been reported that the JAK/STAT pathway is active in granulosa cells. Leptin suppresses anti-Mullerian hormone (AMH) mRNA through the JAK2/STAT3 pathway in granulosa cells of women undergoing IVF (39). Additionally, prolactin (PRL) up-regulates the expression of endogenous bone morphogenetic protein-(BMP) and Smad1/5/8 signaling activity by inhibiting Smad6 expression via the JAK/STAT pathways, which leads to suppression of PRL receptor signaling in rat granulosa cells (40). In COC, the JAK/STAT pathway modulates the expression of key COC expansion related genes, such as *Has2*, *Ptgs2*, *Tnfaip6*, and *Ptx3* (21). Our results, together with those from previous reports, suggest that the JAK/STAT pathway plays an important role in normal ovarian function, including the expression of LHR.

Since a number of previous studies have repeatedly emphasized that IL-6 negatively affects steroidogenesis, the presence of IL-6 was assumed to be of pathophysiological relevance. However, IL-6 may induce LHR mRNA in the presence of FSH activation, and this may have important physiological effects on follicular growth. On the other hand, since many other factors are reported as essential local factors of follicular growth, the relationship between IL-6 and these other factors remains to be clarified.

In this study, we demonstrated that IL-6 increases FSH-induced expression of LHR and LHR mRNA by enhancement of gene transcription in rat granulosa cells. We also found that IL-6 activity is mediated by the JAK/STAT pathway, and these results suggest that IL-6 and FSH work cooperatively to regulate ovarian function.

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