Interleukin-6 inhibits the expression of luteinizing hormone receptor mRNA during the maturation of cultured rat granulosa cells

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Abstract

Interleukin-6 (IL-6) and its receptor components have been shown to be present in rat follicular granulosa cells. The present study was designed to examine the effect of this cytokine on changes in expression of the luteinizing hormone receptor (LHR) messenger RNA and of the steroidogenic enzyme, CYP11A1 (cytochrome P450 scC) in an in vitro model of granulosa cell maturation. Ovarian granulosa cells harvested from immature rats 2 days after treatment with equine chorionic gonadotropin were cultured for 48 h in media containing 10% fetal bovine serum. They were then transferred to a chemically defined serum-free medium and cultured for an additional 72 h. Within 24 h of transfer, the expressions of LHR and CYP11A1 mRNA increased significantly and remained increased for 72 h. The cells responded to exposure to FSH, but not LH, by an increase in production of cAMP before the additional 72 h of culture. The cAMP response to LH was attained within 24 h and persisted for 72 h, whereas the response to FSH decreased continuously with time. Inclusion of IL-6 in the culture medium caused a dose-dependent decrease in expression of LHR mRNA, in addition to a decrease in the cAMP response to LH. Immunoneutralization of endogenous granulosa cell IL-6 resulted in an increase in expression of LHR mRNA, but not CYP11A1 mRNA. The results are consistent with the view that IL-6 may have a physiological role in the maturation of ovarian follicles by modulating the attainment of the LHR in granulosa cells.

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Introduction

Ovarian follicular development and ovulation are dependent upon proliferative and differentiative changes in the granulosa cells (Robker & Richards 1998). The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), not only influence the morphological processes for production of preovulatory follicles, they also have a role in gaining expression of the enzymes needed for steroidogenesis by the thecal cells and granulosa cells (Richards 1994). Particularly important for the latter are CYP11A1 (cytochrome P450 scC), the rate-limiting enzyme for conversion of cholesterol to pregnenolone, and CYP19 (cytochrome P450 aromatase (P450 arom)), the rate-limiting enzyme for synthesis of estradiol from androgen precursors (Goldring et al. 1987, Oonk et al. 1990, Tian et al. 1995). Associated with these enzymic changes, and essential for them, is the expression of LH receptors (LHR) on granulosa cells (Zeleznik et al. 1974, Erickson et al. 1979). Acquisition of these receptors is also a prerequisite for ovulation and differentiation of granulosa cells into luteal cells (Richards et al. 1979, Labarbera & Ryan 1981).

Recently, attention has focused upon secondary ovarian paracrine factors that influence follicular development (Erickson & Danforth 1995). These include growth factors, mostly insulin-like growth factor I (IGF-I), and cytokines such as interleukin-1β (IL-1β) that can modulate gonadotropin receptor expression and ovulation. Their importance is exemplified by the finding that both male and female IGF-I null mice are infertile (Baker et al. 1996). Follicular development in these null mice is deficient, and even pharmacologic doses of gonadotropin fail to induce ovulation. Furthermore, in vitro studies have shown that IGF-I enhances FSH-stimulated expression of the LHR in rat granulosa cells (Kanzaki et al. 1994). Expression of these receptors in cultured rat granulosa cells was also markedly suppressed by IL-1β (Gottschall et al. 1988, 1989).

Another cytokine, interleukin-6 (IL-6), usually associated with regulation of B and T cells in the immune system, has been found in the reproductive tract. Rat ovarian epithelium (Ziltener et al. 1993), granulosa cells (Gorospe et al. 1992) and luteal tissues (Tellier et al. 1998) have been shown to produce IL-6. Bioactive IL-6 was also found in the fluid of preovulatory human follicles.
(Machelon et al. 1994). The IL-6 receptor consists of two parts, an α chain (CD126) that has affinity for IL-6 but no signalling capacity, and a β chain (gp130; CD130) that has no binding affinity for IL-6, but is responsible for signal transduction (Taga et al. 1989). The presence of both components of IL-6 receptor, in addition to IL-6, have been reported to be present in cultured rat granulosa cells (Tamura et al. 2000). The latter study showed that IL-6 inhibited the production of estrogen by granulosa cells that had been stimulated by FSH. An earlier study (Gorospe et al. 1992) found that IL-6 inhibited FSH-stimulated, but not basal, progesterone production in in vitro rat granulosa cells.

The mechanism(s) of IL-6 action in the ovary, particularly regarding the maturation and differentiation of granulosa cells has not been investigated. The present study was therefore designed to investigate the role of IL-6 in regulating the expression of LH receptor mRNA, and the gonadotropic response of rat granulosa cells in an in vitro model.

Materials and Methods

Hormones and reagents

Ovine FSH (NIDK oFSH–19-SIAFP: AFP-4117A) and ovine LH (NISDK oLH26: AFP-5551B) were obtained from Dr A F Parlow of the National Hormone and Pituitary Program (Harbor/UCLA Medical Center, Torrance, CA, USA). Porcine collagen type I (Cellmatrix) and human extracellular matrix, which was comprised of laminin IV and heparan sulfate proteoglycan (HSPG), were acquired from Waco Pure Chemical Co. (Tokyo, Japan). Androstenedione, bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Murine IL-6 and IL-1α were supplied by the Imamichi Institute for Animal Reproduction (Ibaraki-ken, Japan). Murine IL-6 and IL-1α were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrocortisone, transferrin and albumin were purchased from Nitta Gelatin (Osaka, Japan) and Collaborative Biomedical Products (Bedford, MA, USA) respectively. Hydrocortisone, transferrin and albumin were purchased from Nitta Gelatin (Osaka, Japan) and Collaborative Biomedical Products (Bedford, MA, USA) respectively. Hydrocortisone, transferrin and albumin were purchased from Nitta Gelatin (Osaka, Japan) and Collaborative Biomedical Products (Bedford, MA, USA) respectively. Hydrocortisone, transferrin and albumin were purchased from Nitta Gelatin (Osaka, Japan) and Collaborative Biomedical Products (Bedford, MA, USA) respectively.

Animals and cell preparation

Immature (21-day-old) female Wistar strain rats were supplied by the Imamichi Institute for Animal Reproduction (Ibaraki-ken, Japan). Animals were maintained in quarters controlled for temperature (23 ± 1°C), humidity (55 ± 5% relative humidity) and light (12 h light/day) and given free access to food and water. All procedures were performed in accordance with institutional guidelines for experimental animal care in the Tokyo University of Pharmacy and Life Science. Animals (24-day-old) were injected s.c. with 20 IU equine chorionic gonadotropin (eCG) (Teikoku Hormone MFG Co., Tokyo, Japan) dissolved in saline. Twenty-four hours later, ovaries were removed and granulosa cells harvested as described previously (Davis et al. 1989, Tamura et al. 2000). Cultures were started in collagen Type I-coated dishes at a density of 1–2 × 10^5 cells/well and maintained for 2 days in 0·5 ml Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) plus 100 µg/ml gentamycin, and grown to subconfluency. For the induction of granulosa cell maturation and differentiation, a serum-free culture medium was used, supplemented with 5 µg/ml transferrin, 40 ng/ml insulin, 100 ng/ml FSH and 10 ng/ml LH. Granulosa cells maturation was monitored by the increase in cAMP production in response to LH or FSH, as described previously (Lindsey & Channing 1979, Asakai et al. 1995).

Cyclic AMP assay

After an incubation of 3 h in serum-free DMEM medium, phosphodiesterase activity was inhibited by replacing the medium with 150 µl DMEM containing either FSH (50 ng/ml) or LH (250 ng/ml) in the presence of 3-isobutyl-1-methylxanthine 400 µM. Incubation was continued for 1 h and the medium then collected for assay. The amount of cAMP released into the medium was determined using the Biotrak cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech UK Limited, Amersham, Bucks, UK) as described before (Tamura et al. 2000).

Analyses of LH receptor mRNAs by RT-PCR

Poly(A) RNA was isolated using a Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech, Piscataway NJ, USA). The rat cDNA clone for the LHR was kindly provided by Dr Deborah L Segaloff (University of Iowa College of Medicine, Iowa City, IA, USA). A digoxigenin-labeled LHR cDNA probe corresponding to bases 10–622 was prepared for Southern blot using a labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) and following the manufacturer’s instructions. RT-PCR with LHR mRNA was performed using a one-step kit (AMV, TaKaRa, Shiga, Japan) with 30 cycles (30 s at 94°C, 30 s at 55°C and 2 min at 72°C). Oligonucleotide primers that were successfully used for the RT-PCR reaction (Zhang et al. 1994) were synthesized (sense primer: 5’-CGAGTCGACGATCTGAGAA-3’, antisense primer: 5’-ACACGGACTGAGGAGA-3’). The predicted lengths of the LHR mRNA fragments amplified by RT-PCR are 953 bp. A similar analysis was performed to examine CYP11A1 mRNA levels (sense primer: 5’-CTGGAAGGTGTAGCTACG-3’ and
antisense: 5'-GTACTTGCTGAAGTCTCGCT-3'). RT-PCR data for β-actin were used for standardization as an internal control, as described before (Tamura et al. 2000). For Southern blot analysis for LHR, the PCR products were subjected to electrophoresis on a 1·2% agarose gel, transferred to nylon membranes, and fixed by baking. After the membranes had been prehybridized for 3 h at 45°C in high-SDS buffer, hybridization was performed for 18 h at 45°C with high-SDS buffer containing the digoxigenin-labelled cDNA probes (25 ng/ml). The membranes were washed twice at 65°C for 15 min with 0·1% SDS–2 × SCC and then incubated with alkaline phosphatase-labeled antidigoxigenin antibody (75 mU/ml; Boehringer Mannheim) followed by detection with a chemiluminescent substrate (CDP-Star: New England Nuclear, Boston, MA, USA).

Statistics

The results of EIA and densitometric analysis are presented as the means ± s.e.m. and examined by one-way ANOVA with Fisher’s protected least significant difference test. Differences with P<0·05 are considered significant.

Results

Effect of the chemically defined medium on gonadotropin-stimulated cAMP production and on expression of mRNAs for LHR and CYP11A1 (P450 scc)

To characterize the in vitro granulosa cell maturation model, we first examined the changes in cAMP production induced by exposure to FSH or LH during 72 h of culture in a chemically defined serum-free medium. Subconfluent cells, which had been cultured for 2 days in DMEM containing 10% FBS, responded to FSH with a significant increase in cAMP production, but there was no response to LH (Fig. 1A, 0 h). However, an enhanced response to LH was obtained within 24 h of transfer to the serum-free medium and this was maintained for 72 h. Concomitant with the attainment of sensitivity to LH, there was a loss in response to FSH, so that by 72 h it was barely above the control value (Fig. 1A, 72 h). The serum-starved cells quickly increased expression of LHR and CYP11A1 (P450 scc) mRNAs (Fig. 1B). Increased expression in both was at or near maximum by 24 h and remained increased for 72 h. A similar pattern of changes was observed in the expression of CYP 11A1 (P450 arom) mRNA.

Effect of IL-6 on LHR mRNA expression in the in vitro granulosa cell maturation model

Subconfluent cells were incubated in the chemically defined medium in the presence or absence of IL-6 (10 ng/ml) for 0–72 h and the level of LHR mRNA examined (Fig. 2A). LHR mRNA expression, which was increased by removal of serum from the medium (Fig. 1B) was markedly reduced by IL-6. Figure 2B shows representative data for the effect of increasing doses of IL-6 on LHR mRNA after 72 h of culture. Quantitation of the dose-dependent inhibition by IL-6 is shown in Table 1. Inhibitory effects of IL-6 on the expression of LHR were confirmed by northern analysis using cRNA probe (data not shown).
Effect of IL-6 on LH-induced cAMP production

In order to examine whether the LHR was influenced functionally by IL-6 treatment, the FSH- or LH-induced cAMP response was examined (Fig. 3). After subconfluent cells were cultured for 72 h in the chemically defined serum-free medium, LH-induced, but not FSH-induced, cAMP production was significantly enhanced (Fig. 1). Addition of IL-6 into the medium had no effect upon basal production, but significantly decreased the LH-stimulated cAMP production after 72 h of culture (Fig. 3A). This inhibition of LH response by IL-6 was dose-dependent (Fig. 3B). Included in Fig. 3B is the result of exposing the cells to 10 ng/ml of IL-1β. An inhibitory effect on cAMP concentrations similar to that seen with IL-6 was observed with this cytokine, which also has been shown to decrease the number of LHR in granulosa cells (Gottschall et al. 1989).

Effect of immunoneutralization of endogenous IL-6 secreted from cultured granulosa cells

To examine the role of endogenous IL-6, which has been shown (Gorospe et al. 1992, Tamura et al. 2000) to be secreted by granulosa cells, in the expression of LHR, granulosa cells were cultured for 72 h in the absence or presence of anti-IL-6 IgG. Treatment with the anti-IL-6 IgG (10 µg/ml) enhanced LHR mRNA expression by 72 h, compared with cells incubated with nonimmune IgG, although no such effect was observed after 24 h of culture (Fig. 4). However, anti-IL-6 IgG did not affect the expression of CYP11A1 (P450 scc) mRNA levels (Fig. 4), which was different from the inhibitory effect of this antibody on CYP19 (P450 arom) (Tamura et al. 2000).

Discussion

The effect of IL-6 on the acquisition of LHR expression by developing granulosa cells was examined using an in vitro model of granulosa cell maturation. We show

**Table 1**

<table>
<thead>
<tr>
<th>IL-6 (ng/ml)</th>
<th>LHR mRNA</th>
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<tbody>
<tr>
<td>0</td>
<td>1·00 ± 0·004</td>
</tr>
<tr>
<td>1</td>
<td>1·05 ± 0·127</td>
</tr>
<tr>
<td>3</td>
<td>0·79 ± 0·159</td>
</tr>
<tr>
<td>10</td>
<td>0·39 ± 0·110*</td>
</tr>
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*P < 0·05 compared with IL-6 (0 ng/ml).
that IL-6 attenuates the expression of LHR mRNA, in addition to the LH responsiveness for cAMP production in cells cultured in a serum-free medium. We have recently confirmed (Tamura et al. 2000) that, in addition to IL-6 mRNA, cultured granulosa cells express mRNAs encoding the components of the IL-6 receptor complex – that is, the ligand-binding α subunit and the signal transducing gp130 chain. These findings prompted us to investigate IL-6 as a possible autocrine/paracrine regulator of LHR expression. In the in vitro culture system, granulosa cells secrete about 2 ng/ml of bioactive IL-6 per 24 h, which implies a possible physiological role for this cytokine in ovarian function.

Analysis of the 5′ region of the rat LHR gene revealed that rat LHR gene seems to be constitutively inhibited by 5′ upstream sequences and that the promoter activity is regulated by at least three additional DNA domains that bind multiple trans-factors in a tissue-specific manner (Dufau et al. 1995). Competition between inhibitory and neutral DNA binding factors within upstream and promoter domains (C-box) appears to be important for the regulation of LHR gene expression. It is generally accepted that homodimerization of gp130 induced by IL-6 activates two signal transduction pathways: the STAT3/APRF pathway and the MAP kinase cascade (Kishimoto et al. 1995). Activation of JAK kinase induces tyrosine-specific phosphorylation of APRF/STAT3 and its nuclear translocation. Activation of an unidentified tyrosine kinase triggers the activation of Ras-MAP kinase cascades followed by the activation of NF-IL-6, which is a
transcription factor, highly homologous to C/EBP (Akira et al. 1990, 1995). Although there is no information regarding NF-IL-6 expression in the ovary, C/EBPβ mRNA is abundant in the granulosa cells of preovulatory follicles. Furthermore, the ovaries of C/EBPβ-deficient mice lack corpora lutea (Sterneck et al. 1997), suggesting that this transcription factor is essential for the final differentiation of granulosa cells into luteal cells. We have not examined whether IL–6 influences transcription rates, the stability of transcripts or the possible mechanism(s) by which it could do so. Attenuation of LHR mRNA levels by IL–6 in chemically defined medium–treated cells may be due to decreased gene transcription or mRNA stability, or both. There is no clue regarding direct interactions between the 5′-flanking region of LHR and IL–6–inducible transcription factor NF–IL–6. There certainly is evidence for regulation of mRNA stability by hormones or cytokines (Nielsen & Shapiro. 1990). Zhang et al. (1998) have suggested that the increase in LHR mRNA by IGF–1 in murine Leydig tumor cells is mainly due to increased mRNA stability. Lu et al. (1993) have observed that the decrease in steady-state LHR mRNA levels by human choric gonadotropin (hCG) in pseudopregnant rats was not associated with a decrease in the transcription rate of the gene, but rather to a significant decrease in the half–life of the receptor transcripts. Therefore IL–6 might decrease the synthesis of protein(s) that stabilize the transcripts or increase the synthesis of those that destabilize them.

IL–6 is produced by many different cell types and has a wide variety of physiological functions. Increasing evidence has emerged indicating that IL–6 plays an autocrine or paracrine role in the regulation of several reproductively important functions. For example, IL–6 stimulates the release of anterior pituitary hormones such as adrenocorticotropic hormone, prolactin, growth hormone and LH in rats (Spangelo et al. 1989, 1995) and regulates the secretions of placental lactogen (Stephanou & Handwerger 1994) and hCG (Nishino et al. 1990) in human trophoblast cells. Inhibitory effects of IL–6 on ovarian steroidogenesis (Gorospe et al. 1992, Tamura et al. 2000) have been reported for rats. This cytokine also may be involved in endometrial proliferation and differentiation (Tabbibzadeh et al. 1989). In previous studies in our laboratory, IL–6 was shown to reduce the expression of CYP19 mRNA during granulosa cell maturation (Tamura et al. 2000). Ample evidence has been presented to show that expression of this enzyme, in addition to the LHR, are essential for the preovulatory maturation of the follicle and its ovulation (Richards 1994). The present results provide evidence that IL–6 down–regulates the expression of LHR in the process of in vitro granulosa cell maturation. This cytokine may have an important inhibitory role in the regulation of follicular maturation that is related to final stages of granulosa cell differentiation in preovulatory follicles.

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