Original Paper

IL-1 α Up-Regulates IL-6 Expression in Bovine Granulosa Cells via MAPKs and NF- κ B Signaling Pathways

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Key Words

IL-6 • Granulosa cells • IL-1 α • MAPKs pathway • NF- κ B pathway

Abstract

Background/Aims: IL-6 is one of the main cytokines in regulating ovarian follicular development and ovulation. However, the factors that regulate IL-6 expression in follicles are still unclear. The aim of this study was to elucidate the mechanisms underlying the effect of IL-1 α on IL-6 expression in granulosa cells. **Methods:** IL-6 expression after IL-1 α with/without inhibitors treatment was analyzed by RT-qPCR and ELISA. The phosphorylation of proteins induced by IL-1 α was analyzed by western blot. The intracellular cAMP level was assayed by immunoassay kit. **Results:** IL-1 α has a dose-dependent effect on IL-6 expression in granulosa cells. This promoting effect can be significantly attenuated by Erk, c-Jun, p38 and I κ B proteins inhibitors, respectively. Moreover, the phosphorylation levels of Erk, c-Jun, p38 and I κ B α proteins were significantly increased after IL-1 α treatment. In addition, we also found that IL-1 α not only reversed the cAMP attenuated IL-6 expression, but also increased IL-1 α mRNA expression in granulosa cells. **Conclusion:** The regulation of IL-1 α on IL-6 expression is mediated by activation of MAPKs and NF- κ B signaling pathways. Moreover, IL-1 α may regulate the ovulation-related genes expression in granulosa cells by an autocrine and/or paracrine manner.

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Introduction

Female fertility depends on the normal development and growth of ovarian follicles and eventually leads to ovulation. These processes are precisely regulated by many genes, including follicle stimulating hormone, luteinizing hormone and follicle derived steroid hormones [1, 2]. In addition, many local factors, including inflammatory cytokines play

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an important regulatory role in the ovarian cycle, especially in ovulation [3-5]. Moreover, it is also involved in the occurrence and development of ovulatory disorder infertility [6-8]. Inflammatory cytokines not only play a biological effect by themselves, but also have a complex regulatory relationship between each other, both at the gene level and the protein level.

IL-6 is produced by various types of cell, such as leukocytes, keratinocytes, endothelial cells, fibroblasts, and some tumor cells. It often displays hormone-like characteristics that affect homeostatic processes in many systems, including the reproductive system [3, 9, 10]. Similar to the effects in the inflammatory response, IL-6 also plays a dual role in regulation of follicular development and ovulation. It not only suppresses steroidogenesis induced by FSH [11, 12], but also promotes the LH receptor expression [13] and cumulus-oocyte complexes expansion [14]. Although previous studies have confirmed that IL-6 and its receptors expressed in granulosa cells [15-17], the factors that regulate IL-6 expression in ovarian follicles are still unknown.

IL-1 is another important type of inflammatory cytokines, mainly from the activated monocytes/macrophages and epithelial and endothelial cells. It induced expression of pro-inflammatory cytokines and chemokines through activating mitogen-activated protein kinase (MAPKs) and nuclear factor-kappa B (NF- κ B) signaling pathways by binding to IL-1 receptor type 1 (IL-1R1) on the cell membrane [18-20]. IL-1 is also one of the most important cytokines in local ovarian follicles [21]. It involved in several ovulation associated events such as synthesis of proteases, regulation nitric oxide and prostaglandin production [22]. On the other hand, previous studies has also found that hCG-induced ovulation rates were decreased in rats after treatment with IL-1 receptor antagonist (IL-1Ra) [23,24]. Although IL-1 α and IL-1 β are encoded by different genes, they exert similar biological effects by binding to the same receptor (IL-1R1). However, IL-1 α is more able to rapidly initiate the inflammatory cytokine expression both in the mature and precursor forms when binding to IL-1R1, and cause the aseptic inflammation, which has many similarities with ovulation [25]. It remains unclear whether IL-1 α can modulate the expression of IL-6 in granulosa cells.

In this study, we examined the effects of IL-1 α on IL-6 mRNA expression and protein production in granulosa cells. Moreover, the mechanisms of its action were also studied. The results showed that IL-1 α could up-regulates the IL-6 expression via MAPKs and NF- κ B signaling pathways. The regulatory effects of IL-1 α on ovarian granulosa cells may be achieved through an autocrine and/or paracrine manner.

Materials and Methods

Antibodies and reagents

Recombinant bovine IL-1 α was purchased from Kingfisher Biotech. U0126, SP600125, SB203580 and BAY11-7082 were obtained from Beyotime Biotechnology. Erk antibody, p-Erk antibody, c-Jun antibody, p-c-Jun antibody, I κ B α antibody and p-I κ B α antibody were purchased from Cell Signaling Technology. p38 antibody, p-p38 antibody and β -actin antibody were purchased from LifeSpan BioSciences. Forskolin were purchased from Abcam.

Granulosa cell cultures

Bovine ovaries were collected from a local abattoir and brought to the laboratory in saline at 30-35°C within 2 h after euthanasia. Healthy follicles greater than 8mm in diameter were separated after washed with sterile PBS three times. Ovarian follicles were cut in half, and then the follicle walls were washed with serum-free cell culture medium. Granulosa cells were collected by briefly centrifuge and washed three times with the culture medium. The cells were counted and assessed for viability using trypan blue staining. Granulosa cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, USA) supplemented with 1g/L bovine serum albumin (BSA; Sigma, USA), 1% nonessential amino

acids(Gibco,USA), 1% insulin-transferrin-selenium (ITS; Gibco,USA) in a humidified atmosphere under 5% CO₂ at 37°C.

Quantitative real-time PCR

The granulosa cells were cultured in 6-well plates containing 1×10⁶ viable cells in 2ml cell culture medium. The fresh medium was replaced 24h before inhibitors and IL-1 α treatment. Total RNA was extracted from granulosa cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was generated from 1 μ g of total RNA by using the PrimeScript RT reagent kit (Takara, China). Real-time PCR was performed in a total volume of 25 μ l containing 12.5 μ l SYBR premix Ex Taq II, 1 μ l forward primer (10 μ M), 1 μ l reverse primer (10 μ M). The primers involved in the experiment were synthesized by Beijing Genomics Institute. The sequences of all primers used in this work are as follows: IL-6(forward,5'-ATGCTTCCAATCTGGGTCAATC-3';reverse,5'-ACTCGTTCTGGAGGTAGTCCAGGTA-3'),IL-1 α (forward,5'-CCTCTCTCTCAATCAGAAGTCC-3';reverse,5'-CCACCATCACCACATTCTCC-3'),GAPDH(forward,5'-GATGGTGAAGTCGGAGTGAAC-3';reverse,5'-GTCATTGATGGCGACGATGT-3'). Relative quantifications of mRNA were performed using the 2^{- $\Delta\Delta$ CT} comparative method. RNase free dH₂O was used as the negative control reaction.

Measurement of IL-6 secretion by granulosa cells

Granulosa cells were cultured in 24-well plates containing 1×10⁵ viable cells in 1ml cell culture medium. The supernatant was collected and centrifuged at 24h after the IL-1 α with or without inhibitors treatment. These samples were stored at -80°C until assayed. IL-6 secreted by granulosa cells was measured by ELISA kit (Abcam, UK). Absorbance was measured at 450 nm using a microplate reader. The concentration of IL-6 in the samples was calculated by comparison with the standard curve.

SDS/PAGE and western blot analyses

Granulosa cells were cultured in 60-mm dishes and incubated with IL-1 α when the cells reached subconfluence. The cells were washed with pre-cooled PBS and then lysed with RIPA lysis buffer. The protein concentrations were determined by the BCA protein assay after the cell lysate centrifugation. The lysate (20 μ g) was then resolved on 12% SDS/PAGE gels and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, the primary antibody was incubated with the protein antigen transferred to the PVDF membrane. After sufficient binding, secondary antibodies were combined with corresponding primary antibody. The immune complexes were visualized by the enhanced chemiluminescence reaction.

Detection of intracellular Cyclic AMP levels

Granulosa cells were treated with different concentrations of IL-1 α when the cells reached subconfluence. Competitive immunoassay kit (Abcam, UK) was used to determine of intracellular Cyclic AMP (cAMP) level in cells. Briefly, removed the media from adherent cells and add enough 0.1M HCl to cover the bottom of the plate. The cells were incubated in 0.1M HCl for 10min at room temperature. Cell debris was removed by centrifugation after cells were uniform lysed. The supernatant were stored at -80°C until assayed. Absorbance was measured at 405nm using a microplate reader. The intracellular cAMP level in the samples was calculated by comparison with the standard curve.

Data analysis

All data shown are means $\pm$ SEM from three independent experiments. Comparisons between groups were performed by one-way ANOVA. The significant differences between the control group and each treated group were determined using the Tukey test. A value of P<.05 was considered significant.

Results

IL-1 α promotes IL-6 expression in granulosa cells

To investigate the effect of IL-1 α on IL-6 biosynthesis, we analyzed the mRNA expression and protein production of IL-6 in granulosa cells. Granulosa cells derived from large follicles

were cultured in 6-well plates and treated with different concentrations of IL-1 α (0-10ng/ml). As shown in Fig. 1 A, IL-1 α has a dose-dependent effect on IL-6 mRNA expression. The expression levels of IL-6 mRNA were significantly increased after treatment with higher concentrations of IL-1 α (3ng/ml and 10ng/ml) compared with lower concentration (0-1ng/ml). The change trend of IL-6 protein production in culture supernatant was consistent with mRNA expression in granulosa cells after IL-1 α treatment (Fig. 1 C).

IL-1 α induced IL-6 expression is attenuated by inhibitors of MAPKs and NF- κ B signaling pathways

It is known that the IL-1 can induce synthesis and secretion of inflammatory cytokines through MAPKs and NF- κ B signaling pathways [16]. However, little is known about the biological function of IL-1 α in local follicles. To identify the signaling pathways involved in IL-1 α up-regulation of IL-6 expression in granulosa cells, we analyzed IL-6 expression in the absence or presence of U0126, SP600125, SB203580 or BAY11-7082. As shown in Fig. 1 B and Fig. 1 D, the promoting effect of IL-1 α on the expression of IL-6, both at the gene level and protein level in granulosa cells was significantly attenuated by the corresponding inhibitor of MAPKs and NF- κ B signaling pathway compared with the control group (IL-1 α at 10ng/ml).

IL-1 α induces phosphorylation of Erk, c-Jun, p38 and I κ B α proteins

To further verified the role of MAPKs and NF- κ B signaling pathways in IL-1 α up-regulation of IL-6 expression in granulosa cells, we used western blot analysis the protein

Fig. 1. Effects of IL-1 α on IL-6 expression in granulosa cells. A and C, Granulosa cells were treated with different concentrations of IL-1 α (0-10ng/ml), and the mRNA expression levels and protein production levels of IL-6 were analyzed at 3h or 24h using RT-qPCR or ELISA, respectively. B and D, Granulosa cells were pretreated with Erk inhibitor U0126 (10 μ M), c-Jun inhibitor SP600125 (10 μ M), P38 inhibitor SB203580 (10 μ M) or I κ B α inhibitor BAY11-7082 (10 μ M) for 1h. Cells were then treated with IL-1 α (10ng/ml) for 3h or 24h. IL-6 mRNA and protein production levels were measured by RT-qPCR or ELISA, respectively. Results were expressed as the mean $\pm$ SEM of at least 3 independent experiments. Values without a common letter were significantly different ($P < .05$).

Fig. 2. Effects of IL-1 α on phosphorylation of Erk, c-Jun, p38 and I κ B α proteins in granulosa cells. A, B, C and D, The subconfluent granulosa cells were exposed to IL-1 α (10ng/ml) for 5 or 15 min. Whole-cell lysates were used for Western blot to detect levels of phosphorylated Erk (p-Erk), phosphorylated c-Jun (p-c-Jun), -phosphorylated p38 (p-p38) and phosphorylated I κ B α (p-I κ B α). The detection of Erk, c-Jun, p38 and I κ B α proteins served as a loading control. The phosphorylation level of the protein is expressed as the ratio of the phosphorylated protein to the total protein. Results were expressed as the mean $\pm$ SEM of at least 3 independent experiments. * (P < .05) versus control group (5min). # (P < .05) versus control group (15min).

phosphorylation induced by IL-1 α (10ng/ml) at 5min and 15min. The results showed that the phosphorylation level of Erk after IL-1 α treatment was significantly higher than the control group at 15min (Fig. 2 A and Fig. 2 E). We also found that the phosphorylation levels of c-Jun and p38 were significantly increased after exposure of IL-1 α , either at 5min or 15min (Fig. 2 B, C, F and G). In addition, the I κ B α phosphorylation was significant increased at 15min after IL-1 α treatment compared with the control group (Fig. 2 D and Fig. 2 H).

cAMP attenuated IL-6 expression is reversed by high concentration of IL-1 α

It is known that cAMP is involved in the regulation of many cell functions, for example, regulation of cytokine synthesis and secretion [26-28]. Here, we examined the intracellular

Fig. 3. Effects of IL-1 α on intracellular cAMP production in granulosa cells. Granulosa cells were treated with different concentrations of IL-1 α (0-10ng/ml), and the concentrations of intracellular cAMP were measured by ELISA at 1.5h (A) and 3h (B), respectively. Results were expressed as the mean $\pm$ SEM.

Fig. 4. Effects of cAMP on IL-6 mRNA expression in granulosa cells. Granulosa cells were pretreated with Forskolin (10 μ M), a cAMP specific agonist, for 1h. Cells were then treated with different concentrations of IL-1 α (0.1-10ng/ml) for 3h. IL-6 mRNA levels were measured by RT-qPCR. Results were expressed as the mean $\pm$ SEM of at least 3 independent experiments. Values without a common letter were significantly different (P < .05).

Fig. 5. Effects of IL-1 α on IL-1 α mRNA expression in granulosa cells. Granulosa cells were treated with different concentrations of IL-1 α (0-10ng/ml), and the mRNA levels of IL-1 α were analyzed at 3h using RT-qPCR. Results were expressed as the mean $\pm$ SEM of at least 3 independent experiments. Values without a common letter were significantly different (P < .05).

cAMP levels of granulosa cells after stimulated by different concentrations of IL-1 α (0-10ng/ml) for 1.5h and 3h, respectively. As shown in Fig. 3 A and Fig. 3 B, no significant differences were observed in intracellular cAMP levels between the treatment groups, neither at 1.5h nor 3h. However, we still observed that the intracellular cAMP levels after IL-1 α treatment remained higher than the control group (0ng/ml).

In order to further elucidate the effects of cAMP on IL-6 mRNA expression in granulosa cells, we analyzed IL-6 expression after treatment with Forskolin (10 μ M) and different concentrations of IL-1 α (0.1, 1 and 10ng/ml). As shown in Fig. 4, expression level of IL-6 was reduced by Forskolin treatment alone, although no significant difference in statistics. Moreover, the expression level of IL-6 was significantly decreased after treatment with Forskolin and low concentration of IL-1 α (0.1ng/ml) compared with the control group. But, this attenuating effect can be reversed by high concentrations of IL-1 α (10ng/ml).

IL-1 α promotes IL-1 α mRNA expression in granulosa cells

Many studies have found that the level of IL-1 in follicular fluid and serum sharply increased at preovulatory period, and plays an important regulatory role in ovulation-related gene expression [29-31]. Here, we used different concentrations of IL-1 α (0-10ng/ml) on the granulosa cells from large follicle with the characteristics of pre-ovulation

granulosa cells. The expression levels of IL-1 α mRNA were analyzed after treatment with IL-1 α 3h later. The results (Fig. 5) showed that IL-1 α has a dose-dependent effect on IL-1 α mRNA expression. Interestingly, a dramatic increased in IL-1 α mRNA expression level was observed after treatment with of IL-1 α at 10ng/ml.

Discussion

Inflammatory cytokines is the main cytokine type involved in the regulation of ovarian granulosa cell biological functions. In this study, we discovered that IL-1 α could up-regulate IL-6 expression in granulosa cells derived from large follicles. Moreover, our study further found that MAPKs and NF- κ B signaling pathways involved in this regulation process.

Many studies have shown that cytokines, especially inflammatory cytokines involved in the regulation of follicular development and ovulation. Oocyte-cumulus complexes and granulosa cells were the main regulating objects of inflammatory cytokines [32]. Mutual induction of cytokine gene expression is an important feature of inflammatory cytokines. However, the factors that regulate the expression of IL-6 in the ovarian follicles are still unknown. In this study, we found that the expression levels of IL-6 mRNA and protein in granulosa cells were up-regulated by high concentrations of IL-1 α . It indicated that IL-1 α not only plays directly regulatory roles on the granulosa cells by itself, but also indirectly regulates the biological functions of cells through synthesis of other cytokines. In addition, we also found that IL-1 α could up-regulate the expression of IL-1 α mRNA in granulosa cells. Although IL-1 α can be expressed by granulosa cells, it may also be synthesized by other cells in the ovary. Especially in the preovulatory period, a large number of immune cells are rapidly recruited to the follicles and play a crucial role as facilitators of ovulation [33-35]. It suggests that IL-1 α may regulate the ovulation-related genes expression in granulosa cells by an autocrine and/or paracrine manner.

IL-1 induces cytokine gene expression mainly by triggering MAPKs and NF- κ B signaling pathways. MAPKs signaling pathways involved in the regulation of diverse responses, including female follicular development and ovulation. However, the functions of cytokines will vary with different microenvironment. To clarify the mechanisms underlying the effect of IL-1 α on IL-6 expression in granulosa cells, we specifically blocked the corresponding signaling pathways by using inhibitors. Our data show that up-regulation of IL-6 expression by IL-1 α was attenuated by inhibitors of U0126, SP600125, and SB203580 respectively. In order to further confirm the effect of MAPKs pathways on IL-6 expression after IL-1 α treatment, we detected phosphorylation of Erk, c-Jun and p38 proteins of MAPKs pathway by western blot. The results show that the phosphorylation of signaling proteins was significantly increased after IL-1 α treatment compared with the control group. The above studies indicated that the regulation of IL-1 α on IL-6 expression is mediated by activation of MAPKs signaling pathway. NF- κ B signaling pathway is another important pathway for IL-1 activation in the regulation of inflammatory cytokine gene expression. It not only plays the most important role in the immune system, but also regulates genes expression in many other systems. In this study, we found that IL-1 α -induced IL-6 expression in granulosa cells can be attenuated by NF- κ B inhibitors (BAY11-7082). We also found that the phosphorylation of I κ B α protein was increased after exposure of IL-1 α . It also indicated that NF- κ B signaling pathway is also involved in the IL-1 α -induced IL-6 expression in granulosa cells.

There is an interaction between cAMP and MAPKs signaling pathways in regulating gene expression [36]. It has been reported that cAMP can regulate the biosynthesis of IL-6 in many types of cells [37-39]. Here, we observed that the intracellular cAMP levels after IL-1 α treatment was higher than the control group, although no significant difference in statistics. In order to further clarify the regulatory role of cAMP in IL-1 α -induced IL-6 expression, Forskolin, a cAMP specific agonist, was used to treat cells in combination with IL-1 α . The results showed that cAMP can reduce the IL-6 mRNA expression, and this effect can be reversed by high concentrations of IL-1 α . Inhibitory effect of cAMP on IL-6 expression observed in the present study is consistent with previous study on lung fibroblasts [40]. It

suggests that the cAMP may play a negative role in IL-1 α -induced IL-6 expression in ovarian granulosa cells. In addition, it is also imply that there is a regulatory relationship between IL-1 α , IL-6 and cAMP in processes of the follicular development and ovulation.

In summary, our study demonstrates that IL-1 α can promote IL-6 expression in bovine granulosa cells. This effect is mediated through the MAPKs and NF- κ B signaling pathways. IL-1 α may play a regulatory role in granulosa cells through an autocrine and/or paracrine manner.

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Disclosure Statement

All authors declare that they have no conflict of interest.

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