Original Paper

IL-6 Promotes FSH-Induced VEGF Expression Through JAK/STAT3 Signaling Pathway in Bovine Granulosa Cells

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Key words

IL-6 • VEGF • Granulosa cells • JAK/STAT3 pathway • Angiogenesis

Abstract

Background/Aims: Vascular endothelial growth factor (VEGF) has been demonstrated to play a pivotal role in the regulation of angiogenesis in ovarian follicular development, particularly during the preovulatory period. Although numerous studies have shown that interleukin-6 (IL-6) is one of the major inducing factors that regulate the expression of VEGF in non-ovarian cells, whether it involved in regulating the expression of VEGF in normal ovarian granulosa cells is still unknown. The aim of this study was to elucidate the mechanisms underlying the effect of IL-6 on FSH-induced VEGF expression in bovine granulosa cells derived from large follicles. **Methods:** VEGF mRNA expression in granulosa cells after IL-6 with/without inhibitors treatment was analyzed by RT-qPCR. Phosphorylation levels of ERK1/2 and STAT3 proteins induced by IL-6 were analyzed by western blotting. The protein levels produced by granulosa cells were detected by ELISA. **Results:** High concentration of IL-6 (10ng/ml) can significantly up-regulate FSH-induced VEGF gene and protein expression levels in granulosa cells, and also promote the VEGF upstream regulators HIF-1 α and COX2 mRNA expression. VEGF expression levels were significantly decreased after specifically blocking HIF-1 α and COX2 by using inhibitors. The up-regulation effect of IL-6 on FSH-induced VEGF expression in granulosa cells mainly through activating the JAK/STAT3 signaling pathway, which can be impaired by JAK inhibitors. **Conclusion:** IL-6 can promote FSH-induced VEGF expression in granulosa cells, which is mainly achieved by increasing the expression of HIF-1 α and COX2. This promoting effect is mediated by activating the JAK/STAT3 pathway. Moreover, there may be a synergistic relationship between FSH and IL-6 in the regulation of VEGF expression.

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Introduction

Female fertility depends on the fully development of ovarian follicles until ovulation, which is a continuous process of multi-factor participation. This process is accompanied by a reciprocal cycle of angiogenesis, particularly during the preovulatory period [1]. Newly formed blood vessels can promote the transport of oxygen, nutrients and hormones to the ovaries, and also ensure that different hormones can transfer to target cells [2]. A large number of regulatory factors involved in angiogenesis have been identified and characterized. In particular, vascular endothelial growth factor (VEGF) has been demonstrated to play a pivotal role in the regulation of angiogenesis. VEGF is a specific mitogen for vascular endothelial cells that can directly induce vascular endothelial cell proliferation and formation of blood vessels [3]. In addition to endothelial cells, VEGF gene and protein are also expressed in ovarian granulosa cells, granulosa-lutein cells, thecal cells and thecal-lutein cells [4-6]. It has been found that changes in VEGF expression and its distribution in the ovary are closely related to follicular development and ovulation [7-9].

In addition to regulating the biological function of follicular angiogenesis, VEGF can also increase vascular permeability and promote estrogen and prostaglandin synthesis in ovarian granulosa cells [1, 10]. It is not only involved in the normal physiological processes of ovarian activity, but also involved in the occurrence and development of many ovulatory disorders [11-13]. Numerous factors have been found to regulate VEGF expression, such as hypoxia-inducible factors, cytokines, growth factors and gonadotropins [1, 11, 14-20]. Although numerous studies have shown that inflammatory cytokines are one of the major inducing factors that regulate the expression of VEGF in non-ovarian cells [21-27], whether inflammatory cytokines are involved in regulating FSH-induced VEGF expression in ovarian follicles are still unknown.

IL-6 produced by a variety of cell types, including ovarian granulosa cells [28, 29]. IL-6 exerts the biological function by binding to the IL-6 receptor consisting of IL-6R α and a second protein on the cell membrane. IL-6 first binds to IL-6R α , which subsequently associates with a gp130 dimer. The dimerized gp130 triggers activation of the ERK1/2 and JAK/STAT3 signaling pathways [30]. Many studies have found that IL-6 is involved in the angiogenesis process under physiological and pathological conditions, and this effect may be achieved by promoting the expression of VEGF [31, 32]. Therefore, we speculate that IL-6 may also regulate the expression of VEGF in normal granulosa cells. However, IL-6 is a pleiotropic cytokine that exerts different functions in different microenvironments. Moreover, the biological functions of IL-6 in different cells are also different [33]. It remains unclear whether IL-6 can modulate FSH-induced VEGF expression in ovarian granulosa cells.

In the present study, we examined the effects of IL-6 on FSH-induced VEGF mRNA and protein expression levels and regulatory mechanisms in bovine granulosa cells. The results showed that IL-6 could promote FSH-induced VEGF expression mRNA and protein expression in granulosa cells, which was mainly achieved by up-regulating HIF-1 α and COX2 expression. In addition, we further found that the JAK/STAT3 signaling pathway participates in this regulatory process.

Materials and Methods

Antibodies and reagents

Recombinant bovine IL-6 was purchased from Kingfisher Biotech. U0126, AG490, BAY87-2243, YM155 and NS398 were purchased from Selleck Chemicals. ERK1/2 antibody and phospho-ERK1/2 antibody were purchased from Cell Signaling Technology. Anti-STAT3 antibody and phospho-STAT3 antibody were obtained from LifeSpan BioSciences. β -actin antibody was purchased from Abcam.

Granulosa cell cultures

Bovine ovaries were collected from a local abattoir and brought to the laboratory within 2h after euthanasia. The separation and culture of granulosa cells were carried out according to our previous report

[34]. Briefly, healthy follicles greater from 8mm to 17mm 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 DMEM/F12 (Gibco,USA) supplemented with 1g/L BSA (Sigma,USA), 1% nonessential amino acids (Gibco,USA), 1% insulin-transferrin-selenium (Gibco,USA) in a humidified atmosphere under 5% CO₂ at 37°C.

Extraction of total RNA and preparation of cDNA

The granulosa cells were cultured in 6-well plates containing 1×10^6 viable cells in 2ml cell culture medium. Total RNA was extracted from granulosa cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was generated from 1μg of total RNA by using the PrimeScript RT reagent kit (Takara, China). Briefly, 1μg of total RNA was mixed with 2μl of 5×g DNA Eraser Buffer, 1μl of gDNA Eraser. RNase Free dH₂O was complemented to 10μl, and then incubated at room temperature for 5 min to remove genomic DNA. Followed by addition of 10μl of the reverse transcription reaction solution to carry out the reverse transcription reaction and the resulting cDNA was stored at -20°C for subsequent RT-qPCR.

Quantitative real-time PCR

Quantitative real-time PCR was performed in a total volume of 25μl containing 12.5μl SYBR premix Ex Taq II, 1μl forward primer (10μM), 1μl reverse primer (10μM). 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',IL-6(reverse):5'-ACTCGTTCTGGAGGTAGTCCAGGTA-3';VEGF(forward):5'-CCCACGAAGTGGTGAAGTTCA-3',VEGF(reverse):5'-CCACCAGGGTCTCGATGG-3';HIF-1α(forward):5'-CCATTTTCCACTCAGGACAC-3',HIF-1α(reverse):5'-AATTCATCACTGGTGGCTGT-3';COX2(forward):5'-CCTTTAAGGCTTACCTACTCACCAG-3',COX2(reverse):5'-TGTCAGTGTGTCAGCACATCCAG-3';Survivin(forward):5'-CCTGGCAGCTCTACCTCAAG-3',Survivin(reverse):5'-TAAGTAGGCCAACACGAAAG-3';FSHR(forward):5'-AGCCCCTTGTCACAACCTCTATGTC-3',FSHR(reverse):5'-GTTCTCTCACCGTGAGGTAGATGT-3';GAPDH(forward):5'-GATGGTGAAGGTGGAGTGAAC-3',GAPDH(reverse):5'-GTCATTGATGGCGACGATGT-3'. Relative quantifications of mRNA were performed using the 2^{-ΔΔCT} comparative method. RNase free dH₂O was used as the negative control reaction.

SDS/PAGE and western blotting analyses

Granulosa cells were cultured in 60-mm dishes containing 2.5×10^6 viable cells and incubated with IL-6 and inhibitors when the cells reached subconfluence. The cells were washed with precooled PBS and then lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific). The protein concentrations were determined by the BCA protein assay after the cell lysate centrifugation. The lysate (20μg) was then resolved on 12% SDS/PAGE gels and electrophoretically transferred to a 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.

Measurement of VEGF production by granulosa cells

The granulosa cells were cultured in 24-well plates containing 1×10^5 viable cells in 1ml cell culture medium. The supernatant was collected and centrifuged at 3h after the IL-6 and FSH with or without inhibitors treatment. These samples were stored at -80°C until assayed.VEGF secreted by granulosa cells was detected by bovine VEGF ELISA reagent set (GenWay Biotech, USA). Absorbance was measured at 450nm using a microplate reader. The concentration of VEGF in the samples was calculated by comparison with the standard curve.

Data analysis

Data represent 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 Tukey test. A value of P < 0.05 was considered significant.

Results

IL-6 promotes FSH-induced VEGF expression in granulosa cells

Many studies have found that IL-6 can promote VEGF expression in non-granulosa cells and granulosa cell lines. However, whether it regulates VEGF expression in normal granulosa cells is still unknown. Here, we examined the effect of different concentrations of IL-6 (0.1–10 ng/ml) on FSH-induced VEGF expression in granulosa cells. The results showed that FSH-induced VEGF mRNA expression levels were significantly increased after treatment with any concentration of IL-6. It was also found that higher concentration of IL-6 (10 ng/ml) had a more pronounced effect on FSH-induced VEGF mRNA expression than lower concentration (0.1–3 ng/ml) (Fig. 1A). The change trend of VEGF protein production in culture supernatant was consistent with mRNA expression in granulosa cells after IL-6 treatment (Fig. 3A).

In addition, we also analyzed FSH-induced VEGF expression in granulosa cells at different time (1.5, 3, 6 and 12 h) with the same concentration of IL-6 (10 ng/ml) treatment. The results showed that FSH-induced VEGF mRNA expression levels were significantly increased at 1.5 h and 3 h after IL-6 treatment compared with the control group (1.5 h) (Fig. 1B).

IL-6 promotes FSH-induced HIF-1 α and COX2 mRNA expression in granulosa cells

Previous studies have shown that HIF-1 α , COX2 and survivin are important regulators involved in the expression of VEGF. However, little is known about whether these factors are involved in the regulation process of IL-6 on FSH-induced VEGF expression in granulosa cells. We examined the effect of IL-6 on FSH-induced HIF-1 α , COX2 and survivin mRNA expression in granulosa cells. As shown in Fig. 2A, the FSH-induced HIF-1 α mRNA expression levels were significantly higher than that of the control group after any concentration of IL-6 (0.1–10 ng/ml) treatment, while COX2 mRNA expression level was increased only at 1 ng/ml (Fig. 2B). Although the expression levels of survivin mRNA was significantly higher from that of the control group after treatment with different concentrations of IL-6, there was no significant difference compared with the FSH treatment alone (Fig. 2C). Thus, IL-6 does not up-regulate FSH-induced survivin mRNA expression in granulosa cells.

HIF-1 α and COX2 are involved in the regulation of IL-6 on FSH-induced VEGF expression in granulosa cells

In order to further demonstrate the important role of HIF-1 α and COX2 in IL-6 promotes FSH-induced VEGF expression in granulosa cells, we treated cells with HIF-1 α , COX2 and Survivin inhibitors to specific blocking HIF-1 α , COX2 and survivin, respectively. As shown in

Fig. 1. IL-6 promotes FSH-induced VEGF mRNA expression in granulosa cells. A, Granulosa cells were treated with FSH (50 mIU/ml) and different concentrations of IL-6 (0.1–10 ng/ml) for 3 h. The VEGF mRNA expression levels were analyzed using RT-qPCR. B, Granulosa cells were treated with FSH (50 mIU/ml) or/and the same concentration of IL-6 (10 ng/ml), and mRNA expression levels were analyzed at 1.5, 3, 6 and 12 h using RT-qPCR, respectively. Results were expressed as the mean $\pm$ SEM of at least 3 independent experiments. Values without a common letter were significantly different ($P < 0.05$).

Fig. 2D, the expression levels of VEGF mRNA were significantly lower in the HIF-1 α and COX2 inhibitor-treated groups than in the FSH+IL-6 group. However, the expression level of VEGF mRNA was not significantly altered after survivin inhibitor treatment. The change trend of VEGF protein in culture supernatant was consistent with mRNA expression in granulosa cells after inhibitors treatment (Fig. 3B).

Fig. 2. IL-6 promotes

FSH-induced HIF-1 α and COX2 mRNA expression in granulosa cells. A, B and C, Granulosa cells were treated with FSH (50mIU/ml) and different concentrations of IL-6 (0.1-10ng/ml) for 3h. The mRNA expression levels of HIF-1 α , COX2 and Survivin were analyzed using RT-qPCR, respectively. D, Granulosa cells were pretreated with HIF-1 α inhibitor BAY87-2243 (10 μ M), Survivin inhibitor YM155 (10 μ M) or COX2 inhibitor NS398 (10 μ M) for 1h. Cells were then treated with FSH (50mIU/ml) and/or IL-6 (10ng/ml) for 3h. VEGF mRNA levels were measured by RT-qPCR. Re-

sults were expressed as the mean $\pm$ SEM of at least 3 independent experiments. Values without a common letter were significantly different ($P < 0.05$).

Fig. 3. IL-6 promotes FSH-induced VEGF protein expression levels in granulosa cells. A, Granulosa cells were treated with FSH (50mIU/ml) and different concentrations of IL-6 (0.1-10ng/ml) for 3h, and protein expression levels of VEGF were analyzed using ELISA. B, Granulosa cells were pretreated with HIF-1 α inhibitor BAY87-2243 (10 μ M), Survivin inhibitor YM155 (10 μ M) or COX2 inhibitor NS398 (10 μ M) for 1h. Cells were then treated with IL-6 (10ng/ml) and FSH (50mIU/ml) for 3h. VEGF protein expression levels were measured by ELISA. Values without a common letter were significantly different ($P < 0.05$).

Fig. 4. IL-6 promotes FSH-induced phosphorylation of ERK1/2 and STAT3 proteins in granulosa cells. A, The sub-confluent granulosa cells were exposed to FSH (50mIU/ml) for 5, 15, 30 and 60min. Whole-cell lysates were used for western blotting to detect levels of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated STAT3 (p-STAT3). B, the granulosa cells were exposed to FSH(50mIU/ml) and IL-6(10ng/ml) for 5, 15, 30 and 60min. Whole-cell lysates were used for western blotting to detect levels of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated STAT3 (p-STAT3). The detection of ERK1/2 and STAT3 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. Values without a common letter were significantly different ($P < 0.05$).

Fig. 5. JAK inhibitor attenuates the promotive effect of IL-6 on FSH-induced VEGF expression in granulosa cells. Granulosa cells were pretreated with ERK1/2 inhibitor U0126 (10 μ M) or JAK inhibitor AG490 (10 μ M) for 1h and the cells were then treated with IL-6 (10ng/ml) for 3h. VEGF 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 ($P < 0.05$).

IL-6 promotes phosphorylation of ERK1/2 and STAT3 proteins in granulosa cells

IL-6 activates ERK1/2 and JAK/STAT3 signaling pathways by dimerization of gp130 after binding to IL-6R α on the ovarian granulosa cells membrane. In order to clarify the regulation mechanisms of IL-6 on VEGF expression in granulosa cells, we used western blotting to detect the phosphorylation levels change of ERK1/2 and STAT3 proteins at different time points (5, 15, 30 and 60min) after IL-6 treatment. The results showed that IL-6 could promote FSH-induced phosphorylation of ERK1/2 and STAT3 protein in granulosa cells (Fig. 4).

Fig. 6. FSH and LH promote IL-6 mRNA expression in granulosa cells. A, Granulosa cells were treated with FSH (50mIU/ml) or LH (50mIU/ml) for 3h. The IL-6 mRNA expression levels were analyzed using RT-qPCR. B, Granulosa cells were treated with FSH (50mIU/ml) or/and IL-6 (10ng/ml) for 24h, and FSHR mRNA expression levels were analyzed 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 ($P < 0.05$).

The regulation of IL-6 on FSH-induced VEGF expression in granulosa cells requires the involvement of JAK/STAT3 signaling pathway

In order to further elucidate the regulatory mechanism of IL-6 on FSH-induced VEGF expression in granulosa cells, we used ERK1/2 and JAK inhibitors to specifically block the corresponding signaling pathways. As shown in Fig. 5, the expression levels of VEGF mRNA in AG490 (JAK inhibitors) group was significantly lower than that in control group (FSH+IL-6 group). However, the expression of VEGF mRNA was not significantly changed after U0126 (ERK1/2 inhibitors) treatment.

Gonadotropin promotes IL-6 mRNA expression in granulosa cells

There are complex interrelationships between steroid hormones, cytokines, growth factors and gonadotropin-derived gonadotropins in the local follicles during follicular development and ovulation. In order to clarify the interaction between FSH and IL-6 in regulating the expression of VEGF in granulosa cells, we investigate the effect of gonadotropin on the expression of IL-6 in granulosa cells. As shown in Fig. 6A, Gonadotropin, including FSH and LH, can promote the expression of IL-6 mRNA, whereas IL-6 can also increase the expression of FSHR mRNA in granulosa cells (Fig. 6B).

Discussion

In this study, we found that IL-6 can promote the FSH-induced VEGF expression in bovine granulosa cells, which is mainly achieved by increasing the expression of HIF-1 α and COX2. VEGF mRNA and protein expression levels were significantly reduced after specifically blocking HIF-1 α and COX2 expression by inhibitors. We further found that JAK/STAT3 signaling pathway involved in the regulation of IL-6 on FSH-induced VEGF expression in granulosa cells.

VEGF is not only involved in the occurrence and development of many ovarian diseases, but also plays an important role in the physiological processes, such as follicular development, ovulation and corpus luteum formation [1, 2]. Although a large number of studies have confirmed that inflammatory cytokines play a pivotal role in these biological events in follicular development and ovulation, IL-6 as a major type of inflammatory cytokine, whether it is involved in the regulation of VEGF expression in granulosa cells is unclear. Here, we investigated the regulatory role of IL-6 on FSH-induced VEGF expression in normal granulosa cells. We found that high concentrations of IL-6 can promote FSH-induced VEGF mRNA and protein expression. This is consistent with the results of the study on granulosa

cell lines [11]. Together with previous studies, IL-6 and FSH may have synergistic effect in regulating VEGF expression. In order to further clarify this synergistic effect, we investigated the IL-6 and FSH receptor expression in granulosa cells derived from large follicles after treatment with gonadotropin and IL-6, respectively. The results show that FSH can promote the expression of IL-6 mRNA, whereas IL-6 can also increase the expression of FSHR mRNA in granulosa cells. This result demonstrates that it is possible to amplify the regulatory effect of FSH or IL-6 on cells after IL-6 combined with FSH treatment. It also suggests that IL-6 not only regulates VEGF expression under pathological conditions, but also plays an important regulatory role in physiological conditions.

The expression of VEGF is regulated by a variety of factors. HIF-1 α is a key promoter of VEGF expression in pathological conditions [35-37]. Our study shows that IL-6 can significantly increase the FSH-induced HIF-1 α expression level in normal granulosa cells. In addition, we also found that IL-6 can also promote the FSH-induced COX2 expression level, which is another important gene that regulates VEGF expression in the follicular development and ovulation. It can be speculated that the regulatory effect of IL-6 on FSH-induced VEGF expression may be achieved by inducing the expression of HIF-1 α and COX2. In order to confirm this inference, we used BAY87-2243 (HIF-1 α inhibitor) and NS398 (COX2 inhibitor) to specifically block HIF-1 α and COX2, and found that IL-6 promotes FSH-induced VEGF mRNA expression was significantly decreased. Taken together, the above studies have shown that IL-6 mainly promotes FSH-induced VEGF expression in granulosa cells by inducing the expression of HIF-1 α and COX2.

For granulosa cells, IL-6 regulates the expression of target genes by binding to IL-6 receptors and initiating ERK1/2 and JAK/STAT3 signaling pathways. In order to elucidate the regulatory mechanism of IL-6 promotes FSH-induced VEGF expression in granulosa cells, we used inhibitors to specifically block the corresponding signaling pathway. The results show that FSH-induced VEGF mRNA expression levels were decreased significantly after AG490 (JAK inhibitor) treatment. However, the expression of VEGF mRNA was not significantly changed after U0126 (ERK1/2 inhibitors) treatment. In addition, we also used western blotting to detect changes in intracellular phosphorylation levels after IL-6 treatment. The results showed that IL-6 can significantly increase FSH-induced phosphorylation levels ERK1/2 and STAT3 proteins in granulosa cells. The results once again demonstrated the synergistic effect between IL-6 and FSH in regulating VEGF expression. All of the above studies have shown that JAK/STAT3 signaling pathway is involved in the regulation of VEGF expression in granulosa cells. This is consistent with the results of the study in the granulosa cell lines [11, 37]. In addition to the ERK1/2 pathway, many studies have shown that the JAK/STAT3 pathway plays a key role in different induction factors. Moreover, the biological functions of granulosa cells will be inhibited after specific blocking of the JAK/STAT3 signaling pathway. Our study combined in previous studies shows that JAK/STAT3 pathway plays an important role in regulating follicular development and ovulation, including promoting VEGF gene expression.

Numerous studies have confirmed that inflammatory cytokines play an important regulatory role in ovarian activity [38-40]. In particular, the discovery and application of specific inhibitors and gene knockout animals have more substantiated the previous speculation about the involvement of inflammatory cytokines in follicular development and ovulation. However, the previous and our present study mainly focused on a single type of inflammatory cytokines. The study still lacks the role of cytokine network in ovarian cycle. At present, the biological effects of cytokines in regulating follicular development and ovulation have been recognized, but the mechanisms of the overall regulatory effect in the ovarian cycle remains to be studied.

Conclusion

Our study confirms that IL-6 can promote FSH-induced VEGF synthesis in bovine granulosa cells by increasing the expression of HIF-1 α and COX2. The up-regulation effect

of IL-6 on FSH-induced VEGF expression in bovine granulosa cells mainly through activating the JAK/STAT3 signaling pathway. It is not only conducive to clarify the intrinsic mechanism of IL-6 in regulating ovarian activities, but also help to elucidate the possible mechanism of the occurrence of ovulatory disorders.

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Disclosure Statement

All authors declare that they have no Disclosure Statement.

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