

Role of PPAR γ and its gonadotrophic regulation in rat ovarian granulosa cells *in vitro*

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Abstract

The peroxisome proliferator-activated receptors (PPARs), including PPAR α , PPAR β/δ , and PPAR γ , are a family of transcription factors belonging to the steroid receptor superfamily. In rat ovary, PPAR γ is mainly expressed in granulosa cells of developing follicles, implying a possible role of PPAR γ in ovarian functions. In the present study, the role of PPAR γ and its gonadotrophic regulation in granulosa cells collected from diethylstilbestrol-treated immature rats were studied. The results showed that PPAR γ could inhibit proliferation and induce apoptosis in primarily cultured granulosa cells. PPAR γ could also stimulate the biosynthesis of estradiol and progesterone after FSH pretreatment, and FSH could regulate the functions of PPAR γ through PKA, ERK1/2, and p38 MAPK signaling pathways. These data suggested that PPAR γ may be involved in follicular atresia and FSH-stimulated steroidogenesis during follicle development.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors firstly characterized in the mouse [16]. After that, three PPAR isotypes, named PPAR α (NR1C1), PPAR β/δ (NR1C2), and PPAR γ (NR1C3), were identified [6]. Each PPAR is transcribed from an individual gene and forms a heterodimer with retinoid X receptor (RXR, NR2B). The heterodimer can be activated by both ligands of PPARs or RXR [17]. Ligand binding leads to dissociation of corepressors and recruitment of transcriptional coactivators. Subsequently, the heterodimer binds to PPAR response elements

(PPREs) in the promoter region of target genes and regulates the transcription of target genes [5].

PPARs have been found to be involved in a variety of cellular functions [5, 8], some of which directly influence ovarian physiology. In addition, all three PPAR isotypes have been identified in rat ovary [2]. Different from the other two isotypes, PPAR γ mRNA was localized primarily in the granulosa cells of developing follicles and decreased notably in corpus luteum [18]. A key question remaining, however, is the roles of PPAR γ in the development and functions of granulosa cells.

Developmental state of granulosa cells (ie. cell proliferation, differentiation, and apoptosis) is crucial to follicle development. It has been widely recognized that follicle stimulating hormone (FSH), the prime inducer of ovarian follicle development and granulosa cell differentiation, acts by binding to specific receptors, which were localized exclusively on granulosa cells of developing follicles in the ovary [23]. The reponse of granulosa cells to FSH leads to induced expression of numerous genes and stimulated secretion of estradiol and progesterone [22,13]. Although the interactions between FSH and other signaling pathways are particularly studied because the detailed mechanisms of FSH-induced granulosa cells proliferation and steroidogenesis are unclear so far, the relationship between FSH and PPAR γ has not been studied yet.

To further study the regulatory mechanisms of granulosa cell development and functions, we investigated, in the present study, the roles of PPAR γ in granulosa cell proliferation/apoptosis, and the biosynthesis of estradiol/progesterone, as well as the regulatory mechanisms of FSH on PPAR γ functions using primarily cultured rat granulosa cells.

MATERIALS AND METHODS

Reagents

Dulbecco's modified eagle medium (DMEM) and F12 nutrient mixture (F12) were purchased from Gibco BRL (Gaithersburg, MD). Rosiglitazone, GW9662, Estradiol EIA Kit, and Progesterone EIA Kit were purchased from Cayman Chemical (Ann Arbor, MI). Bovine serum albumin (BSA), diethylstilbestrol (DES), dimethyl sulphoxide (DMSO), androstenedione, BrdU, H89, PD98059 and SB202190 were from Sigma-Aldrich (Oakville, ON, Canada). Anti-BrdU mouse monoclonal antibody and goat-anti-mouse FITC-conjugated secondary antibody were from Zymed (USA). Annexin V Apoptosis Assay Kit was from Baosai Biotech Corp. (Beijing, China). Human follicle stimulating hormone (NIADDK-hFSH-I-3) was obtained from National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIADDK-NIH).

Animals

Sprague-Dawley rats were bred under a controlled environment (temperature maintained at 25°C, lights on from 06:00 to 18:00 h, with free access to food and water) in Experimental Animal Center of Institute of Zoology, Chinese Academy of Sciences. Care and treatment of animals were performed under a protocol approved by the Animal Care and Use Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Granulosa cell culture

Granulosa cells were isolated from DES-primed immature rats with a slight modification of a previously de-

scribed method [12]. 21-day-old female Sprague-Dawley rats were injected i.p. with 2 mg DES in 0.1 ml sesame oil once daily for 3 days. The animals were sacrificed on the fourth day, and the ovaries were quickly removed and placed into culture medium (DMEM:F12=1:1, supplemented with 0.1% BSA (w/v), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin). Granulosa cells were harvested by puncturing the follicles with fine needles (26 gauge) and pressing the follicles gently to expulse the granulosa cells. Cells were recovered by centrifugation (1000 rpm, 10 min) and resuspended with fresh medium. Cell viability was measured by trypan blue exclusion test and the number of viable cells was counted in a hemacytometer. Subsequently, the cells were plated in human fibronectin-precoated culture wells and cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO $_2$. The cells were initially cultured overnight and then the medium was changed to remove nonattached cells. Various test reagents were added and the cells were cultured for indicated periods. In every group of treatment, same number of cells were added to each well, and same volume of culture medium was added. In each experiment, 5–8 mice were sacrificed to collect granulosa cells.

Treatment of the cells

To reveal the functions of PPAR γ , a ligand of PPAR γ (rosiglitazone) were used to activate PPAR γ , and an antagonist of PPAR γ (GW9662) was used to inhibit PPAR γ activity. DMSO was added to the culture medium as vehicle control. For enzyme immunoassay (EIA) and cell apoptosis assays, after culturing cells with GW9662 (10 μ M) for 2 h, the culture medium was replaced by fresh medium with rosiglitazone (10 μ M) and the cells were cultured for additional 24 h, and then the cells and culture medium were collected separately for further assays. For cell proliferation assays, granulosa cells were seeded to coverslips and BrdU was added to the culture medium at a final concentration of 10 μ M for the last 18 h of culture, and the other treatments were same as the above. In FSH-treated cell groups, the exposure to GW9662 was 10 h following 50 ng/ml of FSH treatment, and the subsequent procedures were same as those of FSH-free groups. To study the signaling pathways involved in the regulation of PPAR γ functions by FSH, H89, PD98059, or SB202190, inhibitors to PKA, ERK1/2, or p38 MAPK, respectively, were added to the culture medium 2 h before the addition of FSH. Then, ligand and/or antagonist were added to culture medium after treatment with FSH. All experiments were repeated for three times. In each experiment, 3–4 wells were used for each treatment.

Cell proliferation assays

BrdU incorporation and immunocytochemistry analysis were performed to label cells in the S-phase of cell cycle. After culturing, the cells were fixed in methanol/acetone (1:1) for 10 min and incubated in 2 M HCl

for 45 min to denature DNA. After washed three times in PBS, the cells were neutralized with Na₂B₄O₇ (0.1 M, pH 8.5) for 30 min and washed with PBS for three times. The cells were incubated with anti-BrdU mouse monoclonal antibody at 4°C overnight. The slides were washed with PBS for three times, and then goat-anti-mouse FITC-conjugated secondary antibody was used at 37°C for 1 h. After washed three times with PBS, the cells were stained with propidium iodide (PI) at a final concentration of 5 μ g/ml for 10 min at room temperature and then washed three times with PBS again. Staining was visualized using fluorescence microscope. BrdU positive cells, which showing green fluorescence, were regarded as proliferative cells. PI was used to mark total cells, which showing red fluorescence.

Cell apoptosis assays

Cell apoptosis assays were performed according to manufacturer's protocol. Briefly, after digested with trypsin (0.25%) and harvested by centrifugation, cells were washed with cold PBS for three times, followed by resuspended in cold Binding Buffer and incubated darkly with FITC-conjugated Annexin V for 30 min on ice. The incubation was ceased by adding double volume of Binding Buffer and flow cytometry was performed in 1 h. PI was added 5 min before detection on flow cytometer. The apoptotic cells were marked by FITC-conjugated Annexin V.

Enzyme Immunoassay

The EIA assays for estradiol and progesterone in culture supernatants were performed according to the instructions of the manufacturer. In order to ensure accurate and reproducible results, each assay contains two blank wells, two non-specific binding wells, two maximum binding wells, two total activity wells, and an eight-points standard curve running in duplicate. Each sample was assayed at two dilutions and each dilution was assayed in duplicate. The plates were read at a wavelength of 415 nm when the absorbance of the maximum binding wells were in the range of 0.3–0.8 A.U. (blank subtracted).

Statistical analysis

In cell proliferation assays, the rate of cell proliferation was quantified by counting the percentage of BrdU-positive cells in total cells in the same 5–8 independent areas of each slide. Each experiment contained three independent cultures. In cell apoptosis assays, the rate of cell apoptosis was quantified by measuring the percentage of Annexin V-positive cells in total cells. Values were expressed as means \pm SEM of three repeated experiments and presented in histogram. Statistical analyses were carried out by using the SPSS 10.0 software program (SPSS Inc., Chicago, IL), and differences were considered to be significant at $p < 0.05$ compared to control.

RESULTS

PPAR γ inhibited the proliferation of granulosa cells

In granulosa cells without FSH treatment (Figure 1A, left), rosiglitazone significantly inhibited cell proliferation ($p < 0.01$ compared to control). GW9662 alone had no effect on the proliferation of granulosa cells, but could attenuate the inhibitory effect of rosiglitazone on cell proliferation ($p < 0.05$ compared to rosiglitazone). In FSH-pretreated cells (Figure 1A, right), rosiglitazone failed to inhibit proliferation of granulosa cells.

To investigate the signaling pathways involved in the regulation of PPAR γ activities by FSH, H89, PD98059 or SB202190 were used to inhibit PKA, ERK1/2 or p38 MAPK respectively. After blocking p38 MAPK signaling pathway by SB202190 (Figure 1B, right), rosiglitazone failed to inhibit the cell proliferation. However, after blocking PKA or ERK1/2 signaling pathways by H89 (Figure 1B, left) or PD98059 (Figure 1B, middle) respectively, rosiglitazone inhibited proliferation of granulosa

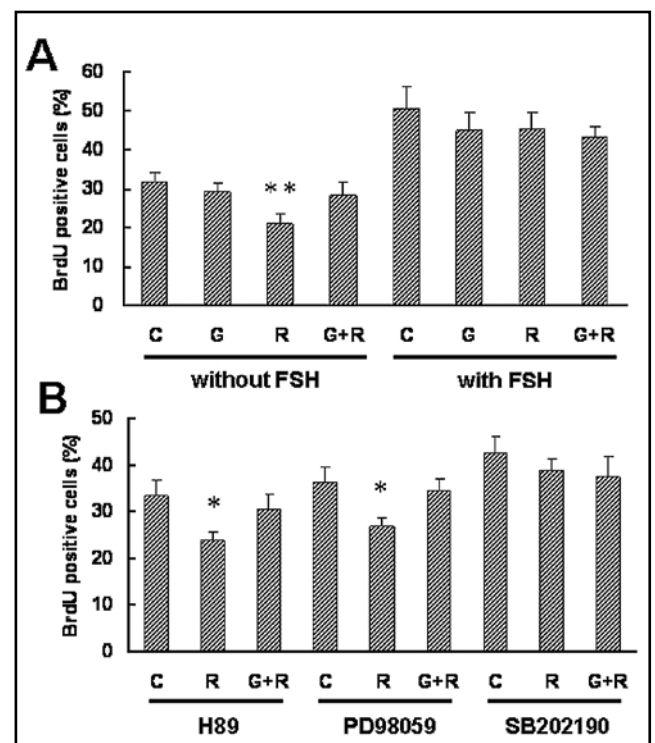


Figure 1. Effects of rosiglitazone and GW9662 on the proliferation of primarily cultured rat granulosa cells. The percentages of BrdU positive cells are presented in histograms. The bars with * or ** are significantly different compared to control at $p < 0.05$ or $p < 0.01$ respectively ($n=3$).

(A) The statistical diagram of BrdU positive cells in the granulosa cells without FSH treatment and after FSH pretreatment;

(B) After inhibition of PKA signaling pathway by H89 (10 μ M), ERK1/2 signaling pathway by PD98059 (10 μ M), or p38 MAPK signaling pathway by SB202190 (10 μ M), the statistical diagram of BrdU positive cells in the presence of FSH.

C, cells treated with DMSO as vehicle control; G, cells treated with GW9662 (10 μ M); R, cells treated with rosiglitazone (10 μ M); G+R, cells treated with rosiglitazone (10 μ M) after pretreatment with GW9662 (10 μ M) for 2 h.

cells in the presence of FSH ($p < 0.05$ compared to control respectively), and GW9662 reversed this effect ($p < 0.05$ compared to rosiglitazone).

PPAR γ induced apoptosis of granulosa cells

In the granulosa cells without FSH treatment (Figure 2A, left), rosiglitazone significantly induced cell apoptosis ($p < 0.01$ compared to control). GW9662 alone had no effect on the apoptosis of granulosa cells, but could attenuate the effect of rosiglitazone on cell apoptosis ($p < 0.05$ compared to rosiglitazone). In FSH-pretreated cells (Figure 2A, right), rosiglitazone failed to induce apoptosis of rat granulosa cells.

To investigate the signaling pathways involved in the regulation of PPAR γ activities by FSH, H89, PD98059 or SB202190 were used to inhibit PKA, ERK1/2 or p38 MAPK respectively. After blocking p38 MAPK signaling pathway by SB202190 (Figure 2B, right), rosiglitazone

failed to induce cell apoptosis. However, after blocking PKA or ERK1/2 signaling pathways by H89 (Figure 2B, left) or PD98059 (Figure 2B, middle) respectively, rosiglitazone induced apoptosis of granulosa cells in the presence of FSH ($p < 0.01$ or $p < 0.05$ compared to their control respectively), and GW9662 reversed this effect ($p < 0.05$ compared to rosiglitazone).

PPAR γ stimulated the biosynthesis of estradiol and progesterone after FSH treatment

EIA were performed to investigate the roles of PPAR γ in biosynthesis of estradiol and progesterone. The results demonstrated that in the granulosa cells without FSH treatment (Figure 3A), rosiglitazone and/or GW9662 had no effect on the concentrations of estradiol or progesterone in the medium. After pretreatment with FSH (Figure 3B), rosiglitazone increased the concentrations of estradiol and progesterone ($p < 0.01$ compared to control), which could be significantly reversed by GW9662 ($p < 0.01$ to rosiglitazone).

In order to study the roles of PKA, ERK1/2 or p38 MAPK signaling pathways in the regulation of PPAR γ activities by FSH, H89, PD98059 or SB202190 were used to inhibit these three signaling pathways respectively. The results showed that after blocking ERK1/2 signaling pathway by PD98059 (Figure 3C, middle), rosiglitazone increased the concentration of estradiol in culture medium in the presence of FSH ($p < 0.05$ to control), and GW9662 reversed this effect ($p < 0.05$ compared to rosiglitazone). However, after blocking PKA or p38 MAPK signaling pathways by H89 (Figure 3C, left) or SB202190 (Figure 3C, right), rosiglitazone failed to stimulate estradiol secretion in the presence of FSH.

DISCUSSION

In rat ovary, the specific expression of PPAR γ in granulosa cells of developing follicles implies a possible role of PPAR γ in ovarian functions. Furthermore, thiazolidinediones (TZDs), a family of synthetic compounds binding to PPAR γ specifically, are now clinically used to treat type II diabetes and some other clinical symptoms frequently associated with insulin resistance, such as polycystic ovary syndrome (PCOS) [9]. All these data raise new questions about the functions of PPAR γ in granulosa cells. To reveal the functions of PPAR γ in granulosa cells, DES was used to stimulate the proliferation of granulosa cells in immature female rats and rosiglitazone, a member of TZDs, was used to activate PPAR γ . DES stimulates granulosa cell proliferation but does not induce differentiation. In primarily cultured granulosa cells from rats treated with DES, rosiglitazone could inhibit proliferation and induce apoptosis, and these effects could be reversed by PPAR γ inhibitor GW9662, suggesting that PPAR γ may be involved in follicular atresia, as extensive granulosa cell apoptosis initiates this process [14]. These results were consistent with those obtained in NIH-3T3 cells [25], showing the

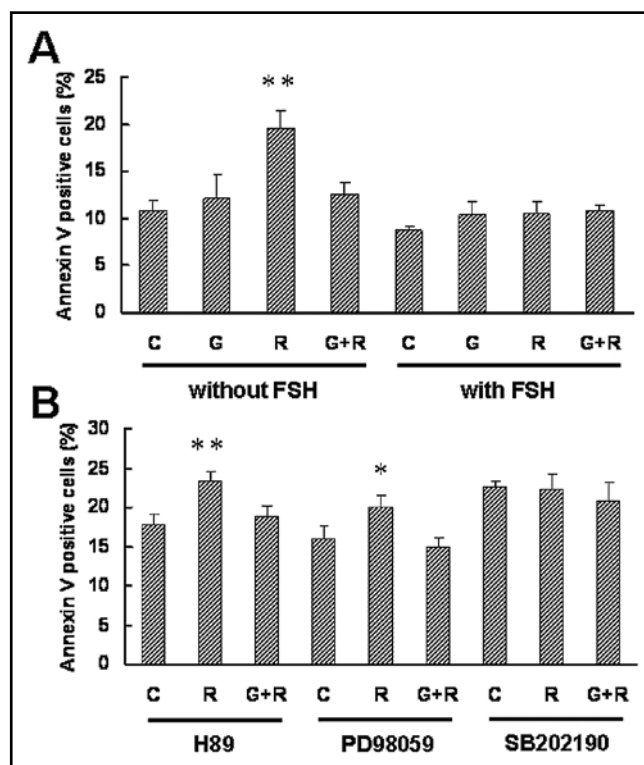


Figure 2. Effects of rosiglitazone and GW9662 on the apoptosis of primarily cultured rat granulosa cells. The percentages of Annexin V positive cells are presented in histogram. The bars with * or ** are significantly different compared to control at $p < 0.05$ or $p < 0.01$ respectively ($n=3$). (A) The statistical diagram of Annexin V positive cells in the granulosa cells without FSH treatment and after pretreatment with FSH (50 ng/ml); (B) After inhibition of PKA signaling pathway by H89 (10 μ M), ERK1/2 signaling pathway by PD98059 (10 μ M), or p38 MAPK signaling pathway by SB202190 (10 μ M), the statistical diagram of Annexin V positive granulosa cells in the presence of FSH. C, cells treated with DMSO as vehicle control; G, cells treated with GW9662 (10 μ M); R, cells treated with rosiglitazone (10 μ M); G+R, cells treated with rosiglitazone (10 μ M) after pretreatment with GW9662 (10 μ M) for 2 h.

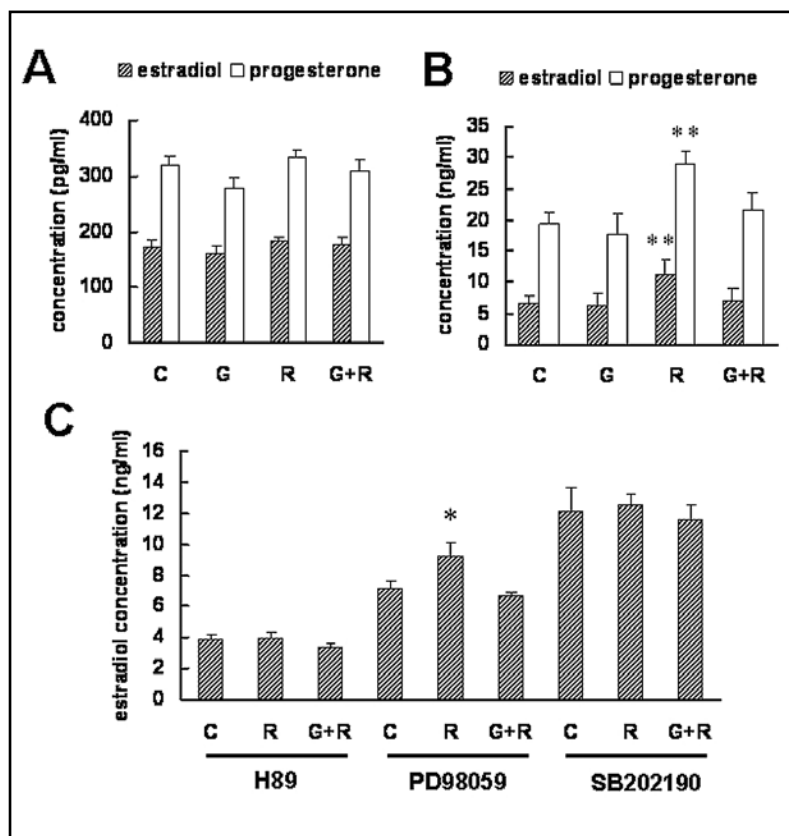


Figure 3. Effects of rosiglitazone and GW9662 on the biosynthesis of estradiol and progesterone in rat granulosa cells. The bars with * or ** are significantly different compared to control at $p < 0.05$ or $p < 0.01$ respectively ($n = 3$). (A) Estradiol and progesterone concentrations of the granulosa cells without FSH treatment; (B) Estradiol and progesterone concentrations of the granulosa cells pretreated with FSH (50 ng/ml); (C) After inhibition of PKA signaling pathway by H89 (10 μ M), ERK1/2 signaling pathway by PD98059 (10 μ M), or p38 MAPK signaling pathway by SB202190 (10 μ M), estradiol concentrations of granulosa cells in the presence of FSH. C, cells treated with DMSO as vehicle control; G, cells treated with GW9662 (10 μ M); R, cells treated with rosiglitazone (10 μ M); G+R, cells treated with rosiglitazone (10 μ M) after pretreatment with GW9662 (10 μ M) for 2 h.

roles of PPAR γ in regulation of cell cycle progression. The roles of PPAR γ in inducing cell cycle withdrawal may be related to inhibition of E2F/DP DNA-binding activity and decrease of Bcl-2 gene expression [1,7]. Molecular mechanisms of PPAR γ in the inhibition of proliferation and induction of apoptosis in rat granulosa cells remain unclear so far.

FSH is a major survival factors for the proliferation and survival of follicular somatic cells [3]. So the interaction between FSH and PPAR γ in the regulation of granulosa cells proliferation and apoptosis is attractive. In granulosa cells without FSH treatment, rosiglitazone could inhibit proliferation and induce apoptosis. After pretreatment with FSH, rosiglitazone had no effect on proliferation or apoptosis of granulosa cells. These results indicated that FSH could regulate the functions of PPAR γ in proliferation and apoptosis of granulosa cells. But how did it achieve? Which signaling pathway(s) is (are) involved? In granulosa cells, FSH could activate not only PKA signaling pathway through cAMP [13], but also ERK1/2 and p38 MAPK signaling pathways [4, 20]. In order to study the roles of these signaling pathways in the regulation of PPAR γ functions by FSH, H89, PD98059 and SB202190 were used to inhibit PKA, ERK1/2 and p38 MAPK signaling pathways respectively. The results showed that H89 and PD98059 blocked the inhibitory effect of FSH on the roles of PPAR γ in cell proliferation and apoptosis, suggesting that FSH inhibited the roles of

PPAR γ in proliferation and apoptosis of granulosa cells through PKA and ERK1/2 signaling pathways.

After pretreatment with FSH, activation of PPAR γ could stimulate the biosynthesis of estradiol and progesterone. In cultured granulosa cells collected from PMSG-primed immature rats, activation of PPAR γ can also stimulate the biosynthesis of estradiol and progesterone [18]. These results indicated that PPAR γ may be involved in FSH-stimulated steroidogenesis during follicle development. This would be helpful to clarify the precise mechanism of FSH-stimulated steroidogenesis. However, in cultured human [21] and porcine [10] granulosa cells, activation of PPAR γ inhibited steroidogenesis. These contrary findings may result from the different stages of cell development, different treatment of animals, and/or species variation.

The results of this study also showed that H89 and SB202190 blocked FSH-induced stimulative effect of PPAR γ on estradiol secretion, suggesting that FSH induced PPAR γ to stimulate estradiol secretion through PKA and p38 MAPK signaling pathways. FSH could regulate the functions of PPAR γ in granulosa cell proliferation/apoptosis and steroidogenesis through different signaling pathways. The precise mechanism is currently unknown. PPAR γ can be activated by its ligands [5], including natural ligands (such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ and 9-and 13-hydroxyoctadecadienoic acid) and synthetic ligands (such as insulin-sensitizing

drugs of the thiazolidinedione family). In addition to its ligands, the activity of PPAR γ is also regulated by phosphorylation and cofactors (including corepressors and coactivators) [5,11]. Dissociation from corepressors and interaction with coactivators are necessary for PPAR γ to bind to PPRE [5]. In a ligand-dependent or -independent manner, phosphorylation can increase [26] or decrease [15] the transcriptional activity of PPAR γ under different conditions. Not only PPAR γ , but also RXR and cofactors can be phosphorylated [24,19]. So it is imaginable that these three kinase signaling pathways activated by FSH in granulosa cells could increase or decrease the activities of PPAR γ through different aspects. The detailed mechanisms that FSH regulates the functions of PPAR γ remain to be further investigated.

This research studied the roles of PPAR γ in cultured rat granulosa cells, showing that FSH could regulate the functions of PPAR γ through different signaling pathways. These data is helpful to clarify the mechanisms of folliculogenesis and steroidogenesis, and regulation of PPAR γ functions by FSH.

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