

Effect of testosterone on the expression of PPAR γ mRNA in PCOS patients

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Abstract. Expression of peroxisome proliferator-activated receptor γ (PPAR γ) mRNA in ovarian granulosa cells of patients with polycystic ovary syndrome (PCOS) were explored. Ovarian granulosa cells were extracted from 5 patients with PCOS and 30 normal controls. Expression of PPAR γ mRNA in granulosa cells of the 5 PCOS patients (observation group) and 5 normal controls (control group) was detected by RT-qPCR. The remaining 25 cases of normal human ovarian granulosa cells were cultured *in vitro* for 48 h, followed by cell culture for another 24 h with different concentrations of testosterone, insulin (INS), and rosiglitazone (RGZ). After that, expression of PPAR γ mRNA was detected by RT-qPCR. Relative expression level of PPAR γ mRNA in the observation group was significantly lower than that in the control group. Compared with testosterone concentration at 10^{-2} mol/ml, testosterone concentration at 10^{-3} mol/ml significantly reduced the expression level of PPAR γ mRNA. When the INS concentration was 10^{-5} mol/ml, relative expression level of PPAR γ mRNA was significantly higher than that of the control group ($P < 0.01$). Relative expression level of PPAR γ mRNA was significantly higher than that of the control group when INS concentration was 10^{-4} mol/ml. When the concentration of RGZ was 10^{-4} mol/ml, the relative expression level of PPAR γ mRNA was significantly higher than that of the control group ($P < 0.01$). When concentration of RGZ was 10^{-3} mol/ml, expression level of PPAR γ mRNA was significantly increased comparing to that under a RGZ concentration of 10^{-4} mol/ml or that of the control group ($P < 0.01$). Appropriate concentrations of testosterone can inhibit the expression of PPAR γ mRNA in ovarian granulosa cells, and certain concentrations of INS and RGZ can induce the expression of PPAR γ mRNA in ovarian granulosa cells.

Abnormal expression of PPAR γ mRNA in ovarian granulosa cells may be related to the mechanism of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is mainly characterized by polycystic changes and endocrine disorders in multiple follicular atresia in the ovaries (1). PCOS patients are mainly women of childbearing age and this disease seriously endangers women's physical and mental health. More than 40% of patients with anovulatory infertility have PCOS (2). PCOS can lead to hormonal abnormalities, which are characterized by increase in serum androgens and insulin (INS) and significant reduction in gonadotropins. These hormone disorders can reduce menstrual blood volume or even lead to menopause, hormone obesity, acne, infertility, INS resistant diabetes (3).

Peroxisome proliferator-activated receptor γ (PPAR γ) activates gene transcription and regulates the expression of downstream target genes. Its ligand is called peroxisome proliferator. PPAR γ activates peroxisomes after binding to peroxisome proliferator and exerts a series of biological effects (4,5). PPAR γ exists in various tissues such as fat, small intestine, and ovarian tissues, and plays an important physiological role in the process of cell growth, production of steroid hormones, remodeling of tissues, and metabolism of glucose and lipids (6,7). PPAR γ is downregulated by luteinizing hormone (LH). In recent years, many studies have shown that androgens may also regulate PPAR γ expression. Activated PPAR γ regulates the secretion of sex hormones and regulate the occurrence, growth, and excretion of follicles (8). Levels and proportions of sex hormones in the body affect ovarian microenvironment, and a good ovarian microenvironment is the basis for the development of high-quality follicles and ovarian growth (9). Aromatase enzymes in ovarian granulosa cells catalyze the formation of androstenedione, testosterone, estradiol (E2) and estrone (10). Keller *et al* (11) found significant upregulation of PPAR γ expression in prenatal androgenic (PA) female monkeys. Rosiglitazone (RGZ) is an agonist of PPAR γ , and its main efficacy is to increase INS sensitivity and reduce INS resistance (12).

In this study, the mechanism of PCOS ovarian function abnormality was explored by detecting the expression of PPAR γ mRNA in PCOS and the expression of PPAR γ mRNA under different concentrations of testosterone, INS and RGZ.

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Materials and methods

Materials

Research subjects. Five patients with PCOS who were treated in Affiliated Hospital of Jining Medical University (Jining, China) from June 2016 to December 2017 and 30 normal controls with non-PCOS who received conventional *in vitro* fertilization embryo transfer were selected. The study was approved by the Ethics Committee of Affiliated Hospital of Jining Medical University, and each patient signed an informed consent.

Inclusion criteria. All PCOS patients met the revised PCOS diagnostic criteria of the American Society of Reproductive Medicine meeting in Rotterdam, The Netherlands, with normal fasting blood glucose and normal spousal semen. PCOS diagnostic criteria: B-scan ultrasonography shows no less than 12 small follicles with a volume of 2-9 mm or large follicles with a volume greater than 10 ml in unilateral or bilateral ovaries; level of serum testosterone is not less than 2.8 nmol/l or androstenedione is not less than 5.3 nmol/l on the 3rd day of menstruation, suggesting the presence of hyperandrogenism; The number of ovulations are reduced or there is non-ovulation. Patients who met two of these criteria were diagnosed as PCOS patients. Females with normal menstrual regular ovulation and normal endocrine function showing no abnormal changes in the abdominal cavity were diagnosed as non-PCOS subjects.

Exclusion criteria. Patients with thyroid glands, adrenal glands and other endocrine disorders, severe hypertension and diabetes, abdominal cavity, pelvic tuberculosis, immune factors inducing premature infertility and previous history of ovarian surgery were excluded.

Methods

Reagents and materials. PBS buffer (Wuhan Procell life science & Technology Co., Ltd., Wuhan, China); Percoll separation solution (volume fraction 50%; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China); collagenase I (Shanghai Cosroma Biotech Co., Ltd., Shanghai, China); M199 medium (10% FBS + 100 U/ml penicillin + 100 µg/ml streptomycin; Wuhan Procell Life Science & Technology Co., Ltd., Wuhan, China); testosterone (Shandong XiYa Chemical Industry Co., Ltd., Shandong, China); INS (Beijing Kuer Chemical Technology Co., Ltd., Beijing, China); RGZ (SinoStandards, Chengdu, China). TRIzol reagent and PowerUp SYBR™-Green Master Mix kit [Thermo Fisher Scientific (China) Inc., Beijing, China]; reproductive hormone detection kits (Beijing Keruimei Technology Co., Ltd., Beijing, China). Primers used were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Collection of follicular fluid and extraction of granular cells. Ovarian granulosa cells were collected from 5 cases of PCOS patients (observation group) and 30 cases of normal people (5 cases were used as control group). Puncture fluid of follicles with a diameter of 18 mm or more was collected. Collected follicular fluid should be clear and free from any menstrual blood contamination. The remaining follicular fluid after collecting eggs was centrifuged at 1,200 x g for 10 min at 4°C, and the supernatant was discarded. The pellet was washed 3 times with 1:1 PBS buffer and resuspended in PBS. An equal volume of Percoll cell separation solution was added and centrifuged at 1,200 x g for 30 min at 4°C. After centrifugation, the liquid was divided into 4 layers. From top to bottom, the

4 layers were supernatant layer (light yellow), granule cell layer (white circle flocculent), separation liquid layer (colorless and transparent), and red blood cell layer, respectively. Granulosa cell layer was aspirated into ammonium chloride solution and vortexed at 37°C. The pellet was retained after centrifugation. Equal volume of 1 g/l collagenase I was mixed with the pellet, followed by incubation at 37°C for 10 min. After filtering with a 300 mesh sieve, the mixture was centrifuged at 1,080 x g for 8 min at room temperature and the supernatant was discarded.

Detection of hormones in observation group and control group. According to ABC ellesa method, 3 ml of fasting venous blood was collected from each participant in observation group and control group in strict accordance with the instructions of the kit.

Culture and treatment of granular cells. A total of 25 cases of human ovarian granulosa cells in the control group were cultured. After washing with PBS 3 times, cells were resuspended in M199 medium and were inoculated into 6-well plates with 10-20 cells in 1 ml medium for each well. Cells were cultured in an incubator (37°C, 5% CO₂). Different concentration of testosterone, INS and RGZ treatment solution was prepared with LM199, and the concentrations of each group were T1 group, 10⁻³ mol/ml and T2 group, 10⁻² mol/ml (13); INS1 group, 10⁻⁵ mol/ml and INS2 group, 10⁻⁴ mol/ml; RGZ1 group, 10⁻³ mol/ml and RGZ2 group, 10⁻⁴ mol/ml (14). After discarding the old culture solution, 1 ml of different treatment solution was added into each well, and 1 ml of LM199 medium was used as blank control. This experiment was performed in triplicate.

RT-qPCR detection of PPARγ mRNA expression. After cell culture had continued for 24 h, cells were washed with PBS three times, and 1 ml of TRIzol was added. The mixture was allowed to stand for 3 min at room temperature and mixed by pipetting. cDNA was obtained by reverse transcription using a reverse transcription kit (Beyotime Institute of Biotechnology, Shanghai, China) in a 20 µl reaction system according to the following conditions: 37°C for 45 min and 95°C for 5 min. PCR reactions conditions were: 94°C for 5 min, followed by 35 cycles of 56°C for 45 sec and 72°C for 1 min, and then 72°C for 10 min. GAPDH was used as an endogenous control and the primer sequences are 5'-AGAGATGCCATTCTGGCC-3' (forward) and 5'-GTGGAGTAGAAATGCTGGAGA-3' (reverse). Upstream and downstream primers for PPARγ are: 5'-CAGGAGCAGAGCAAAGA-3' and 5'-TGGTGAAGACGC CAGTGGGA-3', respectively. 2^{-ΔCq} method was used to statistically process RT-qPCR results (15).

Statistical analysis. SPSS19.0 [AsiaAnalytics (formerly SPSS China), Shanghai, China] statistical package was used for all statistical analysis. Measured data were expressed as mean ± standard deviation. Comparisons between two groups based on the distribution characteristics were performed using two independent samples t-test, and comparisons among multiple groups were performed using ANOVA analysis and LSD test, as a post hoc test. Enumeration data were processed using χ² test. The test significance level is α=0.05.

Results

Basic clinical data of PCOS patients and controls. There was no significant difference in age, sex, BMI, infertility time

Table I. Basic clinical data of PCOS patients and controls.

Variables	PCOS patients (n=5)	Normal people (n=30)	T/ χ^2	P-value
Age (years)	28.97±2.76	29.73±3.62	0.54	0.61
Menstrual cycle (cases)			0.867	0.33
Menstrual period (n, %)	1 (20.00)	6 (20.00)		
Follicular phase (n, %)	2 (40.00)	10 (33.33)		
Luteal phase (n, %)	2 (40.00)	14 (46.67)		
BMI (kg/m ²)	23.75±2.14	22.87±1.97	0.86	0.43
Infertility time (years)	5.24±1.97	4.37±2.56	0.87	0.41
FSH (pg/ml)	5.76±2.11	5.37±1.87	0.39	0.71
LH (pg/ml)	11.76±2.66	5.62±1.47	5.04	<0.001
T (ng/ml)	1.01±0.36	0.32±0.18	4.20	0.01
E2 (pg/ml)	30.82±4.82	31.77±4.49	0.42	0.70
PRL (ng/ml)	17.79±6.10	19.11±5.95	0.45	0.67

PCOS, polycystic ovary syndrome; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; E2, estradiol; PRL, prolactin.

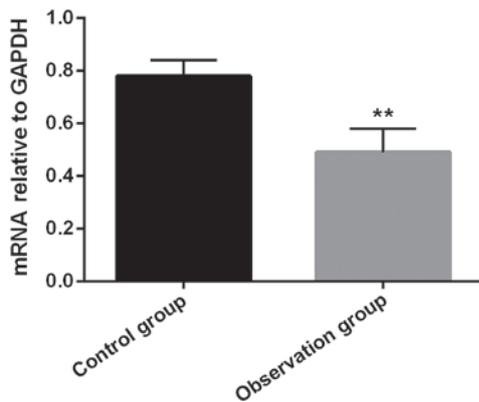


Figure 1. Expression of PPAR γ mRNA in the observation group and control group. RT-qPCR showed that the relative expression of PPAR γ mRNA in ovarian granulosa cells in the observation group was 0.49±0.09, which was significantly lower than that in the control group (0.78±0.06; P<0.01). **P<0.01, compared with the control group. PPAR γ , peroxisome proliferator-activated receptor γ .

and menstrual cycle between 5 PCOS patients and 30 normal controls (P>0.05). Regarding basal endocrinology, levels of LH and testosterone in patients with PCOS were significantly higher than those in the healthy subjects (P<0.01), but there were no significant differences in other three endocrine hormones (P>0.05) (Table I).

Expression of PPAR γ mRNA in the observation and control groups. Relative expression level of PPAR γ mRNA in ovarian

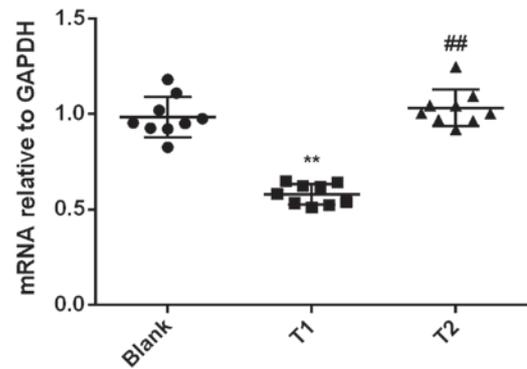


Figure 2. Expression of PPAR γ mRNA under different testosterone treatments. The relative expression of PPAR γ mRNA in T1 group (0.58±0.01) was significantly lower than that in the control group (0.98±0.04) and T2 group (1.03±0.03; P<0.01). There was no significant difference between T2 group and the control group (P>0.05). **P<0.01, compared with the control group. ##P<0.01, compared with the T1 group. PPAR γ , peroxisome proliferator-activated receptor γ .

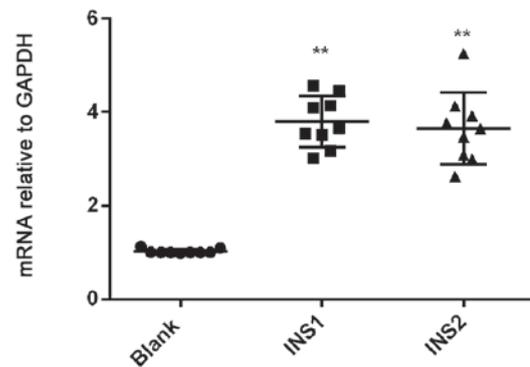


Figure 3. Expression of PPAR γ mRNA under different insulin treatments. The relative expression of PPAR γ mRNA in INS1 group (3.79±0.19) and INS2 group (3.65±0.26) was significantly higher than that in the control group (1.02±0.02; P<0.01). There was no significant difference in PPAR γ mRNA expression between INS1 group and INS2 group (P>0.05). **P<0.01, compared with the control group. PPAR γ , peroxisome proliferator-activated receptor γ .

granulosa cells in the observation group was 0.49±0.09, which was significantly lower than that in the control group (0.78±0.06; P<0.05) (Fig. 1).

Expression of PPAR γ mRNA in granular cells under the treatment of different concentrations of testosterone. Relative expression level of PPAR γ mRNA in group T1 (0.58±0.01) was significantly lower than that in the control group (0.98±0.04) and T2 (1.03±0.03; P<0.01). However, there was no significant difference between T2 group and the control group (P>0.05) (Fig. 2).

Expression of PPAR γ mRNA in granular cells under the treatment of different concentrations of INS. Relative expression of PPAR γ mRNA in INS1 group (3.79±0.19) and INS2 group (3.65±0.26) was significantly higher than that in the control group (1.02±0.02; P<0.01). However, there were no significant differences in PPAR γ mRNA expression level between the INS1 group and INS2 group (P>0.05) (Fig. 3).

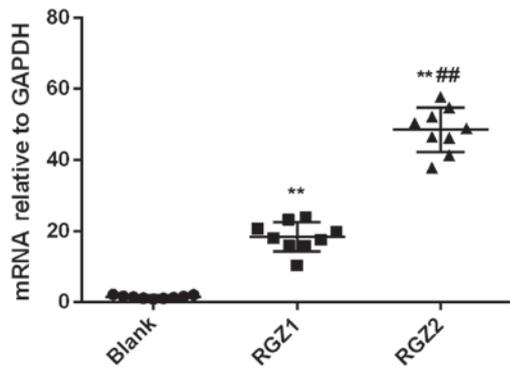


Figure 4. Expression of PPAR γ mRNA under different RGZ treatments. The relative expression of PPAR γ mRNA in RGZ1 group (18.40 \pm 1.39) and RGZ2 group (45.47 \pm 2.09) was significantly higher than that in the control group (1.52 \pm 0.15; $P < 0.01$), and the relative expression of PPAR γ mRNA in RGZ2 group was significantly higher than that in RGZ1 group ($P < 0.01$). ** $P < 0.01$, compared with the control group. ## $P < 0.01$, compared with the RGZ1 group. PPAR γ , peroxisome proliferator-activated receptor γ ; RGZ, rosiglitazone.

Expression of PPAR γ mRNA in granular cells under the treatment of different concentrations of RGZ. Relative expression of PPAR γ mRNA in the RGZ1 group (18.40 \pm 1.39) and RGZ2 group (45.47 \pm 2.09) was significantly higher than that in the control group (1.52 \pm 0.15; $P < 0.01$). Relative expression of PPAR γ mRNA in the RGZ2 group was significantly higher than that in the RGZ1 group ($P < 0.01$) (Fig. 4).

Discussion

PCOS as one of the most common reproductive system diseases among women during childbearing age can easily lead to anovulatory infertility (16). PCOS is mainly manifested as INS resistance and hyperandrogenism. Malignant elevation of androgens inhibits the process of follicle predominance, resulting in sparse or no-ovulation and less menstruation or amenorrhea (17). Peroxidase is one of the major factors in regulating sex hormones and regulates the production, development, and excretion of follicles (18). In this study, we examined the expression of PPAR γ mRNA in PCOS and the expression of PPAR γ mRNA under the treatment of different concentrations of testosterone, INS, and RGZ to explore the possible mechanism of PCOS ovarian dysfunction.

Levels of LH and testosterone in patients with PCOS were significantly higher than those in 30 normal subjects ($P < 0.01$), and there was no significant difference in levels of FSH, E2, and PRL ($P > 0.05$). Compared with normal people, PCOS patients have high levels of testosterone, suggesting the existing of high level androgen in serum of PCOS patients. Multiple follicular atresia in the ovary leads to polycystic changes in the ovary. Excessive androgens beyond the normal range convert to estrone, which in turn negatively affects the hypothalamus-pituitary-gonadal axis and reduces pituitary gonadotropism. This effect can reduce the threshold of pituitary response to gonadotropin-releasing hormone, promote the secretion of LH, promote the production of more androgens, and the excess androgen will aggravate the ovulation disorder and a vicious circle is formed (19,20). Since PPAR γ is downregulated by LH (21), high levels of LH may suggest downregulated PPAR γ mRNA expression in PCOS patients. Therefore, we detected the

expression of PPAR γ mRNA in the observation group and the control group and found that relative expression level of PPAR γ mRNA in the observation group was significantly lower than that in the control group ($P < 0.01$). Therefore, there may be a certain regulatory relationship between testosterone and PPAR γ mRNA expression and PCOS. Subsequently we examined the expression of PPAR γ mRNA under different concentrations of testosterone, INS, and RGZ. Our study found that the relative expression level of PPAR γ mRNA in T1 group was significantly lower than that in the control group and T2 group ($P < 0.01$); however, there was no significant difference between T2 group and the control group ($P > 0.05$), indicating that a certain concentration of testosterone can inhibit PPAR γ mRNA expression, as expected, excess testosterone may downregulate PPAR γ by stimulating LH secretion. However, when the concentration is too high, this inhibitory effect is abolished. This may be because excessively high testosterone activates the negative feedback regulation mechanism of the hypothalamic-pituitary-gonadal axis or reduces the expression of gonadotropin by other means, so as to reduce the secretion of LH. Expression level of PPAR γ mRNA in group INS1 and INS2 was higher than that in the control group ($P < 0.01$). However, there was no significant difference in expression level of PPAR γ mRNA between INS1 and INS2 ($P > 0.05$), indicating that INS has the ability to promote the expression of PPAR γ . Two main characteristics of PCOS patients are hyperinsulinemia and hyperandrogenism, and the expression of PPAR γ is downregulated, indicating that the inhibitory effect of androgen in PCOS patients is greater than that of INS. Relative expression level of PPAR γ mRNA in RGZ1 group and RGZ2 group was significantly higher than that in the control group ($P < 0.01$). Relative expression level of PPAR γ mRNA in RGZ2 group was significantly higher than that in RGZ1 group ($P < 0.01$), indicating that RGZ could induce PPAR γ expression by acting as an agonist of PPAR γ . Münzker *et al* (22) also found that serum testosterone and LH levels were significantly higher in PCOS patients than in normal subjects. Kauffman *et al* (23) found that hormone levels were abnormal in rat model of PCOS and LH and testosterone levels were significantly elevated.

Our study has some shortcomings. Due to the limited resources, the small sample size may affect the results. Hypothalamus-pituitary-gonadal axis has a complex regulatory mechanism. This study found that testosterone can affect the expression of PPAR γ possibly by regulating LH. INS can act on PPAR γ to induce its expression. PPAR γ itself can also affect the secretion of hormones (5,24). Therefore, there may be a complex system of feedback and negative feedback regulation between testosterone, INS, and PPAR γ , and the specific regulatory pathways and signaling pathways remain to be further studied. Single-factor control variable method was used to investigate the effects of different concentrations of testosterone, INS, and RGZ on PPAR γ mRNA expression. However, this is not an applicable method. For example, in patients with PCOS, there are high levels of androgens and INS. Therefore, the combinations of different factors should also be included.

In conclusion, proper concentrations of testosterone can reduce the content of PPAR γ mRNA in ovarian granulosa cells, while PPAR γ mRNA expression can be induced by a certain concentration of RGZ and INS. Expression of PPAR γ mRNA is abnormal in PCOS, which may be involved in the pathogenesis of this disease.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JC drafted the manuscript. JC and GM were mainly devoted to collecting and interpreting the general data. JC and TY were responsible for hormone detection and PCR. All authors read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Affiliated Hospital of Jining Medical University (Jining, China). Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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