Expression of PPARγ and P450arom and possible mechanism of hyperandrogenemia in patients with polycystic ovary syndrome

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Abstract: Objective: To observe the expression of peroxisome proliferator-activated receptor γ (PPARγ) and cytochrome P450 aromatase (P450arom) in granulosa cells and explored the possible pathogenesis of hyperandrogenemia in patients with polycystic ovary syndrome (PCOS). Methods: The study included control group and PCOS group. Control group contained 30 infertile patients without PCOS and PCOS group contained 30 patients with PCOS. Levels of hormones in serum and follicular fluid were determined by electrochemiluminescence method. Levels of PPARγ and P450 arom mRNA in granulosa cells were detected by qRT-PCR. Levels of PPARγ, P450 arom, Smad2 and p-Smad2 protein in granulosa cells were evaluated by Western blot. The correlations of PPARγ and P450 arom with some factors were analyzed. Results: P450arom expression was significantly lower, but PPARγ expression was significantly higher in PCOS group than in control group (P<0.05). PPARγ expression was negatively correlated with P450arom expression (r=-0.547, P=0.001) in PCOS group. p-Smad2 expression was positively correlated with P450arom expression (r=0.530, P=0.003), but negatively correlated with PPARγ expression (r=-0.406, P=0.002) in PCOS group. P450arom expression was positively correlated with estradiol (E2) level (r=0.493, P<0.05), but negatively correlated with testosterone (T) level (r=-0.511, P<0.05) in follicular fluid; and was not correlated with some in-vitro fertilization-embryo transfer (IVF-ET) parameters in patients with PCOS (P>0.05). PPARγ was negatively correlated with E2 level (r=-0.270, P<0.05), but positively correlated with T level (r=0.301, P<0.05) in follicular fluid; and was not correlated with some IVF-ET parameters in patients with PCOS (P>0.05). Conclusion: In granulosa cells of patients with PCOS, PPARγ expression is up-regulated, but P450arom expression is down-regulated. Both PPARγ and P450arom expression are not related to some IVF-ET parameter. Smad2 protein may be involved in the regulatory effect of PPARγ on P450arom.

Keywords: Polycystic ovary syndrome, cytochrome P450 aromatase, peroxisome proliferator-activated receptor γ, p-Smad2, granulosa cells

Introduction

Polycystic ovary syndrome (PCOS), a heterogeneous endocrine disorder, affects 18-20% of women in reproductive age [1]. Hyperandrogenemia, one of the most important endocrine characteristics of PCOS [2, 3], can cause follicular atresia, and lead to anovulation [4]. Therefore, exploring the pathogenesis of hyperandrogenemia has important significance. In granulosa cells, peroxisome proliferator-activated receptor γ (PPARγ) can down-regulate cytochrome P450 aromatase (P450arom) which is a key enzyme to convert androgen into estrogen, leading to high androgen [5]. However, this mechanism has not yet been clear. In some tissues, PPARγ regulates its target genes through TGF-β/Smad signal pathway [6]. It has not been reported how PPARγ down-regulates P450arom in granulosa cells. In addition, there have been different reports on P450arom expression in the patients with PCOS. Some believe that hyperandrogenemia is caused by changes in the substrate of P450arom, but at the same time P450arom activity is normal, even hyperactive [7, 8]. Others hold that the expression or/and activity of P450arom decreases in patients with PCOS [9, 10]. In this study, we further explored the dispute and analyzed the correlations of P450arom with some in-vitro fertilization-embryo transfer (IVF-ET) parameters including number of mature oo-
ocytes and normal fertility rate in the patients with PCOS for the first time.

It is reported that PPARγ can regulate fertility [11, 12]. Faut et al [13] have believed that PPARγ affects pregnancy outcomes because it is associated with apoptosis of antral follicles. Sahmani et al [12] have described that PPARγ level is not associated with the numbers of oocyte retrieval and normal zygotes, and two common polymorphisms of PPARγ can improve fertility rate of IVF-ET [11]. In this study, we determined P450arom, PPARγ and Smad expressions in granulosa cells, analyzed their correlations and explored the possible mechanism that PPARγ regulates P450arom.

Materials and methods

This study involving the use of human tissue specimens was approved by Review Board of the First Affiliated Hospital of Zhengzhou University. The patients' samples used in this study were obtained with informed consent.

Subjects

The subjects in the study were from the patients who underwent IVF-ET in our reproductive center between November 2012 and February 2013. The study included control group and PCOS group. Control group contained 30 infertile patients only caused by tubal factor and PCOS group contained 30 patients with PCOS. PCOS was diagnosed according to revised 2003 consensus on diagnostic criteria related to PCOS. Inclusion criteria for control group were (1) no usage of any hormonal drugs in recent 3 months; (2) normal hormone levels [follicle-stimulating hormone (FSH) <10 IU/L, luteinizing hormone (LH) <10 IU/L, estradiol (E2) <50 pg/ml]; (3) no polycystic changes in ovaries showed by ultrasonography; and (4) regular menstruation and normal ovulation. All subjects in this study had no histories of diabetes, genetic disease, immune infertility, ovarian surgery, uterine malformation and endocrine disease. All subjects in the study underwent IVF and their husbands had normal semen. General data in the two groups are shown in Table 1. There were no significant differences in age, duration of fertility, E2, and Gn duration and dose between the two groups (P>0.05). There was significant difference in LH/FSH, testosterone (T), ovarian volume and number of antral follicles between the two groups (P<0.05) (Table 1).

Controlled ovarian hyperstimulation (COH)

Down-regulation began in midluteal phase by subcutaneous injection of gonadotropin releasing hormone agonist (GnRH-a, Decapeptyl, 0.1 mg/d) for 10 days, and then the dose of Decapeptyl decreased to 0.05 mg/d until administration of human chorionic gonadotrophin (HCG). After attaining the standards of down regulation, gonadotrophin (Gn) was injected. Gn was highly purified recombinant follicle stimulating hormone (r-FSH, 75 IU/ampule) or HMG (75 U/ampule). The dose of Gn was adjusted according to individual status. When type-B ultrasound displayed that the diameter of a dominant follicle reached 18 mm, Gn was stopped and HCG was administered. Oocytes were collected by transvaginal ultrasound-guided puncture 34-36 h later. Insemination, embryo transfer and luteal support were performed according to routine methods used in our reproductive center.

Determination of hormones in serum and follicular fluid

In all patients, 4 ml of venous blood on the second to fourth day of natural menstrual cycle and one milliliter of blood-free follicular fluid on the day of oocyte retrieval were taken for determination of hormone levels using ELISA KIT (Global Biotech, Shanghai, China). For E2, the detection range was 12.35-1000 pg/ml and sensitivity was 4.75 pg/ml. For T, the detection range was 0.1-20 ng/ml and sensitivity was 0.05 ng/ml. For LH, the detection range was 10-200 mIU/ml and sensitivity was 1.27 mIU/ml. For FSH, the detection range was 1.09-70 mIU/ml and sensitivity was 0.7 mIU/ml.

Collection and extraction of granulosa cells

Blood-free follicular fluid was centrifuged at 2000 r/min for 10 min. PBS (12 ml from Hyclone, USA) was added in the cells to prepare single cell suspension. According to 1:1 ratio, the suspension was added into a centrifuge tube containing hydroxypropylmethyl cellulose (Hao Yang Biological Formulation Company, Tianjin, China) followed by centrifugation at 2000 r/min for 30 min. PBS was again added in white cell layer according to 1:1 ratio followed
Polycystic ovary syndrome

Table 1. General data in PCOS group and control group (n=30)

<table>
<thead>
<tr>
<th></th>
<th>PCOS group</th>
<th>Control group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>29.43±3.10</td>
<td>30.43±2.60</td>
<td>0.170</td>
</tr>
<tr>
<td>Duration of infertility (year)</td>
<td>4.85±1.75</td>
<td>5.57±2.30</td>
<td>0.166</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>2.24±1.37</td>
<td>0.59±0.33</td>
<td>0.00’</td>
</tr>
<tr>
<td>Basal E2 (pg/ml)</td>
<td>31.56±11.09</td>
<td>31.78±9.76</td>
<td>0.845</td>
</tr>
<tr>
<td>Basal T (ng/ml)</td>
<td>0.83±0.28*</td>
<td>0.27±0.14</td>
<td>0.00’</td>
</tr>
<tr>
<td>Gn duration (day)</td>
<td>10.08±2.26</td>
<td>11.60±2.32</td>
<td>0.100</td>
</tr>
<tr>
<td>Gn dose (ampoule)</td>
<td>20.24±2.67</td>
<td>21.76±3.18</td>
<td>0.070</td>
</tr>
<tr>
<td>Ovarian volume (ml)</td>
<td>12±8</td>
<td>5±3</td>
<td>0.00’</td>
</tr>
<tr>
<td>No. of antral follicles</td>
<td>29±8.19</td>
<td>12±6.77</td>
<td>0.00’</td>
</tr>
</tbody>
</table>

Notes: PCOS: polycystic ovary syndrome; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estradiol; T: testosterone; Gn: gonadotrophin.

Table 2. Primer sequences used for quantitative RT-PCR in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence primers (5’-3’)</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-γ</td>
<td>Forward: GCCCTTCACTAGTGACTCTCT</td>
<td>193 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATGAGGCTTATGTAGAGCTGAG</td>
<td></td>
</tr>
<tr>
<td>P450arom</td>
<td>Forward: GGACTTTGCCACTGAGTGGAT</td>
<td>164 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCTCTCAACATGATTGGTC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CACGATGGAGGGGCCCGACCTAC</td>
<td>240 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: TAAAGACCTCTATGCCAACACAGT</td>
<td></td>
</tr>
</tbody>
</table>

by centrifugation at 1000 r/min for 10 min. Cells were put in red cell lysis followed by centrifugation at 1000 r/min for one minute. After removal of red supernatant, the sample was immediately stored at -80°C for future use in qRT-PCR and Western blot.

Determination of PPARγ and P450arom mRNA in granulosa cells

Total RNA was isolated from the collected primary granulosa cell using Trizol reagent (Invitrogen, USA) according to manufacturer’s instructions, and was determined through performing a quality analysis testing with Nanodrop Spectrophotometer (samples with a minimum concentration of 10 ng/μL and with an OD 260:280 ratio of 1.8-2.0). First-strand cDNA was created using 2.16 μg of total RNA by reverse transcriptase (Fermentas, Canada). Evaluation of PPARγ and P450arom mRNA levels was achieved by RT-PCR kinetics using PCR machine (EDC-810, Eastwin Life Science Inc, Beijing, China). Reaction conditions were as follows: 94°C 4 min; 94°C 30 s, 56°C 30 s, 72°C 25 s, 30 cycles; 72°C 4 min, 4°C 4 min. The cDNA was then amplified in triplicate using SYBR Green/Flourescein qPCR master mix (Fermentas, Canada) and was detected on an ABI Prism 7900 Sequence PCR machine (Illumina, USA). Reaction conditions were as follows: 50°C 2 min, 95°C 10 min; 95°C 30 s, 60°C 30 s, 40 cycles. The β-actin primer was used as a loading control and the level of mRNA for each gene relative to β-actin was calculated according to the 2^-ΔΔCt cycle threshold method. All primers were provided by Genscript (Hong Kong, China). The specific primers and the size of their amplified fragments are presented in Table 2.

Determination of PPARγ, P450arom, Smad2 and p-Smad2 protein in granulosa cells

Total protein was extracted from granulosa cells. The levels of PPARγ, P450arom, Smad2 and p-Smad2 protein were determined by Western blot according to the instructions of kits (Santacruz, USA). Primary antibodies, rabbit anti human PPARγ and Smad2 antibodies (1:2000) and rabbit anti human P450arom and p-Smad2 antibody (1:1000), were respectively added into 20 μg of total protein, and then were incubated for 24 h followed by washing with PBS three times with each time for 10 min. Secondary antibody, HRP-labeled sheep anti rabbit IgG, was added in the sample, and then was incubated for one hour followed by douching with PBS three times with each time for 5-10 min. Mouse anti human GAPDH was used as internal references. After visualization, grey values were obtained using IIP software. The grey ratio of target band to internal reference band was used as relative level of target protein expression. Testing was performed in triplicate in each group.

Statistical analysis

Statistical treatment was performed with SPSS21.0 software (SPSS, Inc. Chicago, IL, USA). t-test and Pearson correlation analysis were used in the measurement data which were consistent with normal distribution and
homogeneity of variance. Rank sum test and Spearman correlation analysis was used in the data of non-normal distribution. Chi-square test was used in numeration data.

**Results**

*Comparison of general data between the two groups*

There were no significant differences in age, duration of fertility, E2, and Gn duration and dose between the two groups ($P>0.05$). There was significant difference in LH/FSH, testosterone (T), ovarian volume and number of antral follicles between the two groups ($P<0.05$) (Table 1).

*Expressions of protein and mRNA of PPARγ and P450arom in the two groups*

The expressions of protein and mRNA of PPARγ and P450arom in the two groups are shown in Figure 1A-D.

*Correlations of P450arom and PPARγ expressions in granulosa cells with IVF-ET parameters in PCOS group*

This study showed that PPARγ level in granulosa cells was not directly correlated with number...
Table 3. Correlations of PPARγ and P450arom expressions in granulosa cells with E2 and T levels in follicular fluid and IVF-related parameters in PCOS group

<table>
<thead>
<tr>
<th>Items</th>
<th>Correlation coefficient (r)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ expression E2</td>
<td>-0.270</td>
<td>0.019</td>
</tr>
<tr>
<td>T</td>
<td>0.301</td>
<td>0.041</td>
</tr>
<tr>
<td>No. of mature oocytes</td>
<td>0.404</td>
<td>0.223</td>
</tr>
<tr>
<td>Normal fertility rate</td>
<td>-0.028</td>
<td>0.372</td>
</tr>
<tr>
<td>P450arom expression E2</td>
<td>0.493</td>
<td>0.002</td>
</tr>
<tr>
<td>T</td>
<td>-0.511</td>
<td>0.000</td>
</tr>
<tr>
<td>No. of mature oocytes</td>
<td>0.294</td>
<td>0.057</td>
</tr>
<tr>
<td>Normal fertility rate</td>
<td>0.311</td>
<td>0.266</td>
</tr>
</tbody>
</table>

Notes: PCOS: polycystic ovary syndrome; P450arom: cytochrome P450 aromatase; PPARγ: peroxisome proliferator-activated receptor γ; E2: estradiol; T: testosterone; IVF: in vitro fertility embryo transfer.

of mature oocytes (r=0.404, P=0.223) and fertility rate (r=-0.028, P=0.372) (Table 3). In the same, P450arom level in granulosa cells was also not correlated with number of mature oocytes (r=0.294, P=0.057) and normal fertility rate (r=0.311, P=0.266) in patients with PCOS (Table 3).

The study indicated that in PCOS group, P450arom expression in granulosa cells was positively correlated with E2 level (r=0.493, P=0.002), but negatively correlated with T level (r=-0.511, P=0.000) in follicular fluid; on the contrary, PPARγ expression in granulosa cells was negatively correlated with E2 level (r=-0.270, P=0.019), but positively correlated with T level (r=0.301, P=0.041) in follicular fluid (Table 3).

**Expressions of Smad2 and p-Smad2 proteins in the two groups**

Our results indicated although there was no statistical difference in Smad2 protein level between PCOS group (0.398±0.017) and control group (0.408±0.033) (P=0.057) (Figure 2), but p-Smad2 protein level was significantly lower in PCOS group (0.117±0.058) than in control group (0.376±0.684) (P=0.000) (Figure 2B). In the patients with PCOS, PPARγ expression was negatively correlated with P450arom expression in granulosa cells (r=-0.547, P=0.001) (Figure 2A), Smad2 was positively correlated with PPARγ (r=0.304, P=0.271) and P450arom (r=0.093, P=0.741); p-Smad2 was negatively correlated with PPARγ (r=-0.406, P=0.002) (Figure 2C) and positively with P450arom (r=0.530, P=0.003) (Figure 2D).

**Discussion**

Hyperandrogenemia can lead to ovulation failure [14], decreasing pregnancy rate of women in reproductive age. At present, exploring the pathogenesis of hyperandrogenemia has been an important issue in PCOS. Isolated granulosa cells were used in the study. The advantage of primary cells is to retain cellular characteristics in the greatest degree, which allows experimental results to be more consistent with in vivo condition.

**Up-regulation of PPARγ expression and down-regulation of P450arom expression in granulosa cells of patients with PCOS**

P450arom is a key enzyme to convert androgen into estrogen in granulosa cells, so reductions of P450arom expression or activity may be a main cause of hyperandrogenemia in patients with PCOS [15, 16]. There has been considerable debate on P450arom expression level. Some believe that hyperandrogenemia is due to changes in the substrate of P450arom, but at the same time P450arom activity is normal, even hyperactive [7, 17]. Most hold that the expression or/and activity of P450arom decreases in patients with PCOS [9, 10]. In this study, qRT-PCR indicated that P450arom mRNA expression level in granulosa cells was lower in PCOS group than in control group and Western blot also indicated that P450arom protein level in granulosa cells was lower in PCOS group than in control group. These results are consistent with other reports [9, 10], but still remain to be further confirmed by large-sample studies or Meta-analysis.

Peroxisome proliferator-activated receptor (PPAR), a nuclear receptor superfamily, maintains cellular normal function [18]. Likewise, PPARγ plays an important role in PCOS anovulation [19]. PPARγ agonist can inhibit P450-coding CYP19 gene, preventing the conversion of androgen to estrogen [20]. In PCOS group of this study, PPARγ expression was high, P450arom expression was low, and PPARγ was negatively correlated with P450arom in granulosa cells. These results suggest that PPARγ is
involved in the pathogenesis of hyperandrogenemia probably through controlling P450arom.

**Correlations of P450arom and PPARγ expressions in granulosa cells with IVF-ET parameters in PCOS group**

Lazaros et al. [21] have found that P450-coding CYP19 gene is related to reactivity of ovaries to gonadotrophin. They also recently report that allele 7 in CYP19 (TTTA) gene can decrease E2 level and numbers of large ovarian follicles and total ovarian follicles [22]. In ovaries of patients with PCOS, although the number of antral follicles increases, they have dysmaturity. Therefore, we explored the correlations of P450arom expression with number of mature oocytes and normal fertility rate in patients with PCOS in the study for the first time. PPARγ is regarded as an important regulatory factor for fertility [23, 24]. PPARγ may be associated with early apoptosis of antral follicles and further influences pregnancy [13]. However, Shah et al [25] have found that up-regulated PPARγ fails to affect embryo development in mice. Sahmani et al [11] have found that PPARγ protein expression level in granulosa cells is not associated with number of ocyte retrieval and fertility rate, and two common polymorphisms may improve fertility rate in IVF. In patients with PCOS, the correlation between PPARγ expression level in granulosa cells and IVF-related parameters has not yet been reported.

The study indicated that in PCOS group, P450arom expression in granulosa cells was positively correlated with E2 level, but negatively correlated with T level in follicular fluid; on the contrary, PPARγ expression in granulosa cells was negatively correlated with E2 level, but positively correlated with T level in follicular fluid. These results are consistent with other
reports [9, 10]. Our results also displayed that PPARγ level in granulosa cells was not correlated with number of mature oocytes and normal fertility rate; although P450arom level in granulosa cells was positive correlation with number of mature oocytes and negative correlation with fertility rate, they did not exhibited statistical significance in patients with PCOS. These results were consistent with the results reported by Sahmani et al [12].

Pregnancy rate is related to number of transferred embryos and embryo quality, so we did not analyzed the correlations of PPARγ and P450arom with pregnancy rate in this study. Our results displayed that PPARγ and P450arom expression levels in granulosa cells were not related to the number of mature oocytes and normal fertility rate in patients with PCOS. This suggests that PPARγ and P450arom may not directly affect follicular maturation and normal fertilization in patients with PCOS. In future studies, it is necessary to explore effects of PPARγ and P450arom on follicular development, follicular quality, pregnancy rate and IVF-related parameters by dividing patients with PCOS into different groups based on the levels of androgen, P450arom or PPARγ.

**Smad2 protein may be involved in the regulatory effect of PPARγ on P450arom**

PPARγ is related to P450arom, and both are together involved in the pathogenesis of hyperandrogenemia, but the specific mechanism has not yet been clear. Our results indicated that PPARγ expression was negatively correlated with P450arom expression in granulosa cells of patients with PCOS. It has been confirmed that PPARγ can down-regulate P450arom [5], but this regulatory pathway has not yet been clear. It has been reported that in many tissues, PPARγ agonists can inhibit phosphorylation of Smad2/3, and further block TGF-β/Smads pathway, playing a role in anti-fibrosis [26]. Hatanaka et al [27] have also reported that PPARγ agonists block TGF-β/Smads pathway via inhibiting phosphorylation of Smad2/3 in retinal epithelium of monkeys. Ning et al [28] have found that P450arom is a downstream molecule of TGF-β/Smads pathway, and is regulated by the TGF-β/Smads pathway in granulosa cell of mice. Based on reports above, we can see that both PPARγ and P450 are associated with TGF-β/Smads pathway, so we observed Smad2 and p-Smad2 protein expressions in granulosa cells and further analyzed the correlations of Smad2 and p-Smad2 protein with PPARγ and P450arom in the study.

Our results indicated although there was no statistical difference in Smad2 protein level between PCOS group and control group, but p-Smad2 protein level was significantly lower in PCOS group than in control group. These results suggested that Smad2 protein activity was lower in granulose cells of patients with PCOS. Whether low Smad2 protein activity is directly involved in occurrence of PCOS has not yet been clear. To explore the relations among PPARγ, P450arom and p-Smad2 in patients with PCOS, we performed correlation analysis for them. Results indicated that Smad2 was not correlated with PPARγ and P450arom, but p-Smad2 was negatively correlated with PPARγ and positively correlated with P450arom in the patients with PCOS. Based on these results, we infer that Smad2 protein may be involved in the regulatory effect of PPARγ on P450arom.

**Conclusion**

In PCOS granulosa cells, PPARγ expression is up-regulated and P450arom expression is down-regulated. PPARγ is involved in the pathogenesis of PCOS hyperandrogenemia probably through regulating P450arom. However, both PPARγ and P450arom fail to affect the number of mature oocytes and normal fertility rate in patients with PCOS. In future studies, it is necessary to divided patients with PCOS into hyperandrogenemia group and non-hyperandrogenemia group, and to explore the pathogenesis of PCOS hyperandrogenemia.

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**Disclosure of conflict of interest**

None.

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