Original Article
Endometrial progesterone receptor isoforms in women with polycystic ovary syndrome

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Abstract: Context: Polycystic ovary syndrome (PCOS) affects approximately 4%-18% of all reproductive-aged women and is often accompanied by endometrial progesterone (P4) resistance. Endometrial cells from PCOS patients display increased progesterone receptor (PGR) expression; however, in vivo knockout studies and in vitro experiments indicate the two PGR isoforms are not functionally equivalent. Objective: We aimed to compare endometrial PGR isoform expression between non-PCOS and PCOS patients during the proliferative phase. Design: A case-control study. The expression of PGR isoforms (PGRA and PGRB), estrogen receptor alpha (ERα), and markers of cell proliferation was determined by qRT-PCR, Western blot, immunohistochemistry, and immunofluorescence assays. Patient(s): Patients were recruited and diagnosed with PCOS according to the Rotterdam criteria provided by the American Society for Reproductive Medicine and the European Society for Human Reproduction and Embryology. Endometrial biopsy samples were collected from non-PCOS patients with regular menstrual cycles or with hyperplasia (n = 11) and from PCOS patients with or without hyperplasia (n = 14). Result(s): Although the alteration of PGRB mRNA and protein expression was different, we found that PGRA mRNA and protein expression was higher in PCOS patients than non-PCOS patients. PGRA/B and PGRB were localized in both epithelial and stromal cells, with notable changes in the nuclei of epithelial and stromal cells. A similar expression pattern of ERα, vimentin and Ki-67, in association with an increased PGR expression, was observed in PCOS patients. Conclusion(s): These results demonstrated that elevated both PGR isoform expression depends on the presence of PCOS, and our data suggest that abnormal regulation of PGR isoforms is a pathological outcome of defective endometrium in PCOS patients.

Keywords: Progesterone receptor isoforms, estrogen receptor, hyperandrogenism, endometrium, PCOS, infertility

Introduction
Polycystic ovary syndrome (PCOS) is a state of unbalanced steroid hormone production and activity [1] that affects approximately 4%-18% of reproductive-aged women worldwide [2]. Although the exact mechanisms in the pathogenesis of PCOS remain obscure, hyperandrogenism and insulin resistance appear to be the major etiological drivers for reproductive and metabolic abnormalities in PCOS patients [3, 4]. Many clinical studies now provide evidence that the impairment of endometrial function likely causes endometrial hyperplasia and carcinoma, implantation failure, recurrent pregnancy loss, and premature delivery in PCOS patients [5-7]. However, a cause-and-effect relationship between PCOS and endometrial dysfunction-induced infertility has yet to be firmly established. The presence of hyperandrogenism and insulin resistance may be accompanied by additional factors such as abnormal female steroid hormone responsiveness in order for the uterine dysfunction to occur. Therefore, it is important to understand the mechanisms and consequences behind the pathophysiological changes in the endometrium in PCOS patients in order to develop effec-
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tive treatments to prevent endometrial dys-
function-induced infertility.
Under physiological conditions, endometrial
functions are predominantly governed by ste-
roid hormones, estrogen and progesterone
(P4), in most mammalian species [8]. For exam-
ple, 17β-estradiol (E2) drives endometrial cell
proliferation whereas P4 inhibits E2-induced
endometrial cell proliferation [9]. The endome-
trium from PCOS patients is different from nor-
mal endometrium, and it experiences sustained
and persistent estrogen stimulation but mini-
mal or completely absent P4 stimulation [10,
11]. While P4-based oral contraceptives can
inhibit endometrial hyperproliferation [12], and
treatment with megestrol P4 or medroxypro-
gesterone can improve certain cases of endo-
metrial atypical hyperplasia in some PCOS pa-
tients [13], approximately 30% of PCOS patients
fail to respond to a long term, high-dose P4
treatment regimen [14, 15]. P4 resistance
implies a decreased responsiveness of target
tissue to bioavailable P4 [16], and such an
impaired P4 response is seen in the endome-
trium of PCOS patients [17-19]. A question aris-
ing in this context is why in some PCOS patients
normally respond to P4 treatment while in oth-
ers it develops endometrial P4 resistance.
P4 is essential for the initiation and mainte-
nance of pregnancy. All P4 actions are mainly
mediated through binding and activation of the
P4 receptor (PGR), PGRA and PGRB, members
of the superfamily of ligand-induced transcrip-
tion factors [20, 21]. While PGRA and PGRB
display indistinguishable ligand- and DNA-binding
affinities, their actions are remarkably diver-
gent. In vivo knockout studies and in vitro
experiments show that two PGR isoforms are
not functionally equivalent [22, 23]. Several
lines of clinical evidence indicate that the
expression of both PGR isoforms fluctuates in the
normal cycling endometrium in an isoform-
specific and cell-specific manner [24, 25].
Moreover, the elevated PGR expression in
endometrial epithelial cells is greater than that
in endometrial stromal cells in PCOS patients
[26]. These results suggest that one isoform
may be more important than the other in PCOS
patients with P4 resistance. Although high lev-
els of total PGR expression are found in PCOS
patients who have anovulation compared to
PCOS patients who still ovulate [27], the rela-
tive expression pattern of endometrial PGR iso-
forms in PCOS patients has not been described
so far.
This study was designed to investigate PGR iso-
forms and potential molecular markers [estro-
gen receptor alpha (ERα), cytokeratin 8, vimen-
tin and Ki-67] in the endometrial tissues
obtained from non-PCOS and PCOS patients
during the proliferative phase. Using quantita-
tive real-time reverse transcription-polymerase
chain reaction (qRT-PCR), Western blot, immu-
nohistochemical, and immunofluorescent anal-
yses, we obtained mRNA and protein changes
in PGR isoforms in the endometrium to eluci-
de the possible role of PGR isoforms under
PCOS conditions in vivo.

Materials and methods

Study approval

Informed consent was obtained from all pa-
tients in accordance with a protocol reviewed
and approved by the institutional ethical review
board of the Obstetrics and Gynecology Hos-
pital of Fudan University, Shanghai, China
(OGHFU 2013-23).

Patient selection and endometrial tissue col-
lection

Reproductive-aged women (range, 25-45 ye-
ars) with PCOS (n = 14) and without PCOS (n =
11) were originally recruited into the study.
PCOS was diagnosed based on the Rotterdam
criteria provided by the American Society for
Reproductive Medicine and the European So-
ciety for Human Reproduction and Embryology
[28]. All PCOS patients were diagnosed accord-
ing to at least two of the following criteria: 1) ol-
o/anovulation, 2) signs of hyperandrogen-
ism (i.e., hirsutism and acne) and/or biochemi-
cal measurements, or 3) enhanced ovaries (at
least 12 discrete follicles of 2-9 mm in diame-
ter in one ovary or an ovarian volume >10 cm³
observed by transvaginal ultrasonography). All
non-PCOS fertile women taking part in the
investigation had regular menstrual cycles and
showed no evidence of any pathological uterine
disorder. Women with PCOS were excluded
from this study if they had evidence of any
chronic inflammatory disorders such as sys-
temic lupus erythematosus, inflammatory
bowel disease, rheumatoid arthritis, and asth-
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Neither non-PCOS nor PCOS subjects were exposed to any hormonal or steroidal therapies within three months prior to tissues sampling [19, 29]. All endometrial tissues were obtained during the proliferative phase of the menstrual cycle by endometrial curettage of the bisected uteri obtained after hysterectomy for benign gynecological indications. Each endometrial sample was diagnosed and staged by routine pathology analysis based on standard histological criteria [30]. All samples were snap frozen in liquid nitrogen for subsequent RNA and protein analyses or fixed in 10% neutral formalin solution for 24 h at 4°C and embedded in paraffin for histochemical analysis in a non-blinded manner.

RNA extraction and qRT-PCR analysis

For RNA isolation, tissues were lysed using TRIzol Reagent (Life Technologies), and RNA was isolated following standard protocols. qRT-PCR was performed with a Roche Light Cycler 480 sequence detection system (Roche Diagnostics Ltd., Rotkreuz, Switzerland) as previously described [31, 32]. The PCR amplifications were performed with a SYBR green qPCR master mix (#K0252, Thermo Scientific, Rockford, IL). Total RNA was prepared from the frozen whole uterine tissues, and single-stranded cDNA was synthesized from each sample (2 μg) with M-MLV reverse transcriptase (#00-00113467, Promega Corporation, Fitchburg, WI) and RNase inhibitor (40 U) (#00314959, Thermo Scientific). cDNA (1 μl) was added to a reaction master mix (10 μl) containing 2× SYBR green qPCR reaction mix (Thermo Scientific) and gene-specific primers (5 μM each of forward and reverse primers). All primers are indicated in Table 1. All reactions were performed six times, and each reaction included a non-template control. The results for target genes were expressed as the amount relative to the average CT values of ACTB + CYC1 in each sample. Relative gene expression was determined with the 2-ΔΔCT method, and the efficiency of each reaction-as determined by linear regression-was incorporated into the equation. The qRT-PCR results obtained from human endometria were validated by either Western blot analysis or immunohistochemical and immunofluorescence assays as previously described [29, 33].

Histology and immunostaining

Endometrial tissues were fixed in 10% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) staining according to standard procedures. Immunohistochemistry and immunofluorescence were performed according to previously described methods [31, 32]. The endogenous peroxidase and nonspecific binding were removed by incubation with 3% H₂O₂ for 10 min and with 10% normal goat serum for 1 h at room temperature. After incubation with the primary antibody (Table 2) overnight at 4°C in a humidified chamber, the sections were stained using the avidin-biotinylated-peroxidase complex detection system (Vector Laboratories Inc., Burlingame, CA) followed by treatment with 3-amino-9-ethyl carbazole developing reagent plus High Sensitivity Substrate (SK-4200, Vector Laboratories). The sections were imaged on a Nikon E-1000 microscope (Japan) and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden).

Endometrial sections were incubated with primary antibody (Table 2) in 0.01 M tris-buffered saline supplemented with Triton X-100 (TBST) containing 5% nonfat milk overnight at 4°C, and a secondary antibody was applied at room temperature for 1 h. After the sections were washed with TBST, they were re-suspended in mounting medium containing DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories). Sections were examined under an Axiovert 200 confocal microscope (Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 710 META system (Carl Zeiss) and photomicrographed. Background settings were adjusted

<p>| Table 1. Sequences of primer pairs used for qRT-PCR measurement |
|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGR</td>
<td>TTTAAGAGGGCAATGGAAGG</td>
<td>CGGATTITATAACAGATGCAG</td>
<td>74 bp</td>
</tr>
<tr>
<td>PGRB</td>
<td>ATGGGCTGTACCGAGGGG</td>
<td>TCTCAGTCCTCGCTGAGTT</td>
<td>76 bp</td>
</tr>
<tr>
<td>ACTB</td>
<td>CATGACGTCCTACCGCAGGG</td>
<td>CTCTAATGTGACGACAGAT</td>
<td>250 bp</td>
</tr>
<tr>
<td>CYC1</td>
<td>AGCTATCCGTGGTCTCACCC</td>
<td>CCGCATGAACATCTCCATC</td>
<td>225 bp</td>
</tr>
</tbody>
</table>

PGR, progesterone receptor; ACTB, β-actin; CYC1, cytochrome c isoform 1.
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Table 2. Antibodies: species, clone/catalog number, method, dilution, and source

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Cat. No.</th>
<th>kDa</th>
<th>Method</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGRA/B</td>
<td>Mouse</td>
<td>MS197</td>
<td>B116</td>
<td>WB</td>
<td>1:10</td>
<td>Lab Vision (Fremont, CA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IHC</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>PGRB</td>
<td>Mouse</td>
<td>MS196</td>
<td>116</td>
<td>IHC</td>
<td>1:50</td>
<td>Lab Vision</td>
</tr>
<tr>
<td>ERα</td>
<td>Mouse</td>
<td>6F11</td>
<td>66</td>
<td>WB</td>
<td>1:200</td>
<td>Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IHC, IF</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>Cyto 8</td>
<td>Mouse</td>
<td>C5301</td>
<td>52</td>
<td>WB</td>
<td>1:1000</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IHC</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mouse</td>
<td>5G3F10</td>
<td>57</td>
<td>WB</td>
<td>1:250</td>
<td>Cell Signaling Technology (Danver, MA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IHC</td>
<td>1:100</td>
<td></td>
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<tr>
<td>Ki-67</td>
<td>Rabbit</td>
<td>9027</td>
<td></td>
<td>IHC</td>
<td>1:100</td>
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</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>A1978</td>
<td>42</td>
<td>WB</td>
<td>1:2000</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

PGRA/B, progesterone receptor A and B; ERα, estrogen receptor alpha; Cyto8, cytokeratin 8; WB, western blot; IHC, immunohistochemistry; IF, immunofluorescence.

from the examination of negative control specimens. Images of positive staining were adjusted to make optimal use of the dynamic range of detection. All morphological and immunohistochemical assays were performed by at least two researchers in an operator-blinded manner.

Protein isolation and quantitative Western blot analysis

A detailed explanation of the Western blot analysis protocol has been published [31, 32]. Total protein was isolated from whole uterine tissue by homogenization in RIPA buffer (Sigma-Aldrich) supplemented with cOmplete Mini protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostics). After determining total protein by Bradford protein assay, equal amounts (30 μg) of protein were resolved on 4-20% TGX stain-free gels (Bio-Rad Laboratories GmbH, Munich, Germany) and transferred onto PVDF membranes. The membranes were probed with the primary antibody (Table 2) in TBST containing 5% non-fat dry milk followed by HRP-conjugated secondary antibody. When necessary, the PVDF membranes were stripped using Restore PLUS Western blot stripping buffer (Thermo Scientific, Rockford, IL) for 15 minutes at room temperature, washed twice in TBST, and then re-probed. Ultraviolet activation of the Criterion stain-free gel on a ChemiDoc MP Imaging System (Bio-Rad) was used to control for proper loading. Band densitometry was performed using Image Laboratory (Version 5.0, Bio-Rad).

Statistical analysis

GraphPad Prism was used for statistical analysis and graphing. For all experiments, n-values represent the number of individual animals. Data are represented as the means ± SEM. Statistical analyses were performed using SPSS version 24.0 statistical software for Windows (SPSS Inc., Chicago, IL). The normal distribution of the data was tested with the Shapiro-Wilk test. Differences between groups were analyzed by one-way ANOVA, and this was followed by Tukey’s post-hoc test for normally distributed data or the Kruskal-Wallis test followed by the Mann-Whitney U-test for skewed data. All P-values less than 0.05 were considered statistically significant.

Results

Because the two PGR isoforms are products of a single gene (the PGRA sequence is included in the longer PGRB transcript) [22], it is currently impossible to design primers for the detection of the PGRA mRNA alone by qRT-PCR. Our data indicated that endometrial mRNA levels of both PGR isoforms were higher in PCOS patients than non-PCOS patients in the proliferative phase of the menstrual cycle (Figure 1), and Western blot analysis showed that the protein levels of PGRA were also elevated in PCOS patients compared to non-PCOS patients (Figure 2). Additionally, immunohistochemical analysis revealed that although the positive nuclear staining for total PGR and PGRB in the epithelia and stroma was detected in non-PCOS patients, significantly increased immunoreactivity of total PGR and PGRB in the epithelia and stroma was detected in PCOS patients.
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lia and stroma was found in PCOS patients (Figure 3). These results suggested that alterations of endometrial PGR expression in PCOS patients might be accounted for by the overall induction of total PGR protein, particularly by increased PGRA protein expression. In parallel to immunohistochemistry, an immunofluorescence assay for endometrial PGR expression in non-PCOS and PCOS patients further validated that the immunoreactivity of PGR was present in both the intensive nuclei and minimal cytoplasm of endometrial cells (Figure 3).

We and others have shown that the expression of the uterine estrogen receptor (ER) α, cytokeratin 8, vimentin, and Ki-67 proteins are altered in PCOS patients and in PCOS-like rats [32, 34, 35]. To further characterize the endometrial phenotypes in our samples, the expression profiles of several potential molecular markers (ERα, cytokeratin 8, vimentin, and Ki-67) were evaluated. As shown in Figures 2 and 3, although endometrial ERα protein level varied among individuals with no significant changes between non-PCOS and PCOS patients by Western blot analysis, a general increase in ERα protein expression was identified in PCOS patients compared to non-PCOS patients using the immunofluorescence assay. There were no obvious changes in cytokeratin 8 protein levels in non-PCOS and PCOS patients. Concurrently, we observed that increased vimentin expression was associated with increased immunoreactivity of Ki-67 in the glandular epithelia in PCOS patients (Figures 2 and 3).

Discussion

The present study provides the first evidence that the abnormal regulation of endometrial PGR isoform expression is associated with the development of PCOS in women. It is likely that the elevated endometrial PGR isoform expression reflects defective P4 action and an exaggerated influence of estrogen under PCOS conditions. The endometrium from PCOS patients with P4 resistance often displays endometrial hyperproliferation [10]. Although common opinion holds that P4 is the primary regulators of PGR expression and activation [20, 21]; previous studies show that PGR physically associates with ERβ in rat uterine stromal cells [36], and that treatment with estrogen induces PGR isoform expression in human endometrium.
[37]. Our data show that increased PGR isoform expression is associated with elevated ERα expression as well as high levels of cell proliferative factors (vimentin and Ki-67) in the endometrium of PCOS patient. Recently, a similar expression pattern of PGR isoforms and ERα/β is observed in PCOS-like rat uterus [38], and the elevated PGR isoform expression is associated with an increased circulating E2 level [32]. These results suggest that estrogen-ER signaling contributes to the up-regulation of PGR isoforms under PCOS conditions in vivo.

Chronic inflammation has been shown to be closely correlated to high inflammatory mediator production under PCOS conditions [4]. For example, there is a noticeable increase in the inflammatory cytokines such as IL-6 and TNFα in the peripheral circulation of PCOS patients [39, 40]. Importantly, P4 suppresses TNFα-stimulated IL-6 and Mcp-1 production in human endometriotic stromal cells in vitro [41]. Similarly, several studies by our laboratory and others have described increased gene expression of IL-6, TNFα, and Mcp-1 (CCL-2) in PCOS patient endometrium and the PCOS-like rat uterus [31, 35, 42-44]. These findings suggest that the PCOS-induced endometrial inflammation might be inhibited by activation of P4 signaling. However, although several hypothesized mechanisms that might explain how hyperandrogenism and insulin resistance themselves contribute to systemic inflammatory responses [45, 46], interference between the metabolic dysfunction-induced and intrauterine PGR-dependent inflammation under PCOS conditions has not yet been demonstrated. Notably, our experiments combined with qRT-PCR and Western blot analyses provide compelling evidence that the aberrant regulation of uterine PGR isoforms is not only possible, but would likely be PGR-dependent in PCOS-like rats [38].
Therefore, while both PGR isoforms appear to be involved in the uterine inflammatory signaling pathways [47], our results raise the interesting possibility that significant cross talk between PGRA and PGRB might coordinate uterine inflammatory responses in PCOS patients who are lacking P4 and/or suffering from P4 resistance. Because both isoforms function as transcriptional factors leading to the distinct and overlapping signaling pathways needed to maintain uterine function [15, 47], further studies using PGR isoform-specific knockout female mice co-treated with insulin and human chorionic gonadotropin (PCOS-like) will be required to clarify the precise role of individual PGR isoform in the inflammation stages of uterine P4 resistance under PCOS conditions.

The endometrial cycle is divided into menstruation, proliferative phase, and secretory phase, and it is well established that the PGR expression varies with menstrual cycle phases [8, 15]. Our findings suggest that although the patient’s cycle information and tissue histological dating might indicate the proliferative stage for non-PCOS and PCOS patients, different proteomic profiles and regulation, including endometrial PGR isoform expression, exist in the same phase of the menstrual cycle in both non-PCOS and PCOS patients. This means that the day the endometrial sample was collected might create bias in our analysis due to different days of the proliferative phase in the same group patients. In the present study, our qRT-PCR results were validated by either Western blot analysis or immunohistochemical (immunofluorescence) assays. Although the Western blot data are not always in agreement with the immunohistochemical results, we note that these two methods provide different information and the different results might be caused by the use of different patient tissues. We speculate that increased endometrial PGR expression in PCOS patients is mainly caused by increased PGRA expression or is likely due to the different regulatory mechanisms (transcriptional and translational programs) involved.

Our findings also indicate that PGR is present in the cytoplasm of endometrial cells regardless of PCOS conditions. Although endometrial responsiveness to P4 is dependent on nuclear PGR, which is important for our investigation, there is also evidence that classical nuclear PGR can be detected in human endometrial cell cytoplasm using different antibodies [48, 49]. In the absence of an activated ligand, PGR is inactivated through association with various heat shock proteins (hsp), including hsp90, hsp70, and hsp40, and other co-repressor proteins in the cytoplasm [9]. Ligand binding causes release of the multiple hsp-subunit complex, and the PGR undergoes a conformational change that allows the receptor dimer to interact with specific P4 response elements located within the regulatory regions of its target genes [50]. Therefore, it is tempting to speculate from these observations that PGRA and PGRB shuttle between the nucleus and the cytoplasm, with ligand binding inducing interactions between the receptor and nuclear co-activators [51] in the endometrial cells.

Although our patient cohort was small and the results presented here are primarily descriptive, our findings support the link between abnormal regulation of both PGR isoform expression and PCOS-related endometrial dysfunction. Because gene expression analysis of PCOS endometrium reveals P4 resistance and candidate susceptibility genes in PCOS patients [18, 52], further studies are needed to investigate how to modulate the activity of PGR and that of its transcriptional partners and co- regulators for the improvement of P4 resistance-induced endometrial dysfunction in PCOS patients.

Acknowledgements

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