

Original Article

Endometrial progesterone receptor isoforms in women with polycystic ovary syndrome

Min Hu^{1,2}, Juan Li^{1,2}, Yuehui Zhang^{1,3}, Xin Li^{1,4,5}, Mats Brännström⁶, Linus R Shao¹, Håkan Billig¹

¹Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg 40530, Sweden; ²Department of Traditional Chinese Medicine, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, China; ³Department of Obstetrics and Gynecology, Key Laboratory and Unit of Infertility in Chinese Medicine, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin 150040, China; ⁴Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China; ⁵Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China; ⁶Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Sahlgrenska Academy, University of Gothenburg, Gothenburg 41345, Sweden

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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 (PGRα and PGRβ), 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 PGRβ mRNA and protein expression was different, we found that PGRα mRNA and protein expression was higher in PCOS patients than non-PCOS patients. PGRα/B and PGRβ 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 effective

tive treatments to prevent endometrial dysfunction-induced infertility.

Under physiological conditions, endometrial functions are predominantly governed by steroid hormones, estrogen and progesterone (P4), in most mammalian species [8]. For example, 17 β -estradiol (E2) drives endometrial cell proliferation whereas P4 inhibits E2-induced endometrial cell proliferation [9]. The endometrium from PCOS patients is different from normal endometrium, and it experiences sustained and persistent estrogen stimulation but minimal or completely absent P4 stimulation [10, 11]. While P4-based oral contraceptives can inhibit endometrial hyperproliferation [12], and treatment with megestrol P4 or medroxyprogesterone can improve certain cases of endometrial atypical hyperplasia in some PCOS patients [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 endometrium of PCOS patients [17-19]. A question arising in this context is why in some PCOS patients normally respond to P4 treatment while in others it develops endometrial P4 resistance.

P4 is essential for the initiation and maintenance 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 transcription factors [20, 21]. While PGRA and PGRB display indistinguishable ligand- and DNA-binding affinities, their actions are remarkably divergent. *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 levels 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 isoforms in PCOS patients has not been described so far.

This study was designed to investigate PGR isoforms and potential molecular markers [estrogen receptor alpha (ER α), cytokeratin 8, vimentin and Ki-67] in the endometrial tissues obtained from non-PCOS and PCOS patients during the proliferative phase. Using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), Western blot, immunohistochemical, and immunofluorescent analyses, we obtained mRNA and protein changes in PGR isoforms in the endometrium to elucidate the possible role of PGR isoforms under PCOS conditions *in vivo*.

Materials and methods

Study approval

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

Patient selection and endometrial tissue collection

Reproductive-aged women (range, 25-45 years) 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 Society for Human Reproduction and Embryology [28]. All PCOS patients were diagnosed according to at least two of the following criteria: 1) oligo/anovulation, 2) signs of hyperandrogenism (i.e., hirsutism and acne) and/or biochemical measurements, or 3) enhanced ovaries (at least 12 discrete follicles of 2-9 mm in diameter 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 systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis, and asth-

Table 1. Sequences of primer pairs used for qRT-PCR measurement

Gene	Primer	Size
PGR	Forward TTTAAGAGGGCAATGGAAGG	74 bp
	Reverse CGGATTTTATCAACGATGCAG	
PGRB	Forward AATGGGCTGTACCGAGAGGT	76 bp
	Reverse TCTCAGTCCCTCGCTGAGTT	
ACTB	Forward CATGTACGTTGCTATCCAGGC	250 bp
	Reverse CTCCTTAATGTCACGCACGAT	
CYC1	Forward AGCTATCCGTGGTCTCACC	225 bp
	Reverse CCGCATGAACATCTCCCATC	

PGR, progesterone receptor; ACTB, β -actin; CYC1, cytochrome c isoform 1.

ma. 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 μ g) with M-MLV reverse transcriptase (#00-00113467, Promega Corporation, Fitchburg, WI) and RNase inhibitor (40 U) (#00314959, Thermo Scientific). cDNA (1 μ l) was added to a reaction master mix (10 μ l) containing 2 \times SYBR green qPCR reaction mix (Thermo Scientific) and gene-specific primers (5 μ M each of forward and reverse primers). All primers are indi-

cated 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 $2^{-\Delta\Delta CT}$ 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% H_2O_2 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

Table 2. Antibodies: species, clone/catalog number, method, dilution, and source

Antibody	Species	Cat. No.	kDa	Method	Dilution	Source
PGR α /B	Mouse	MS197	B116	WB	1:10	Lab Vision (Fremont, CA)
			A81	IHC	1:50	
PGRB	Mouse	MS196	116	IHC	1:50	Lab Vision
ER α	Mouse	6F11	66	WB	1:200	Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK)
				IHC, IF	1:50	
Cyto 8	Mouse	C5301	52	WB	1:1000	Sigma-Aldrich (St. Louis, MO)
				IHC	1:200	
Vimentin	Mouse	5G3F10	57	WB	1:250	Cell Signaling Technology (Danver, MA)
				IHC	1:100	
Ki-67	Rabbit	9027		IHC	1:100	Cell Signaling Technology
β -actin	Mouse	A1978	42	WB	1:2000	Sigma-Aldrich

PGR α /B, progesterone receptor A and B; ER α , 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 μ g) 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 \pm 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 PGR α sequence is included in the longer PGRB transcript) [22], it is currently impossible to design primers for the detection of the PGR α 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 PGR α 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 epithe-

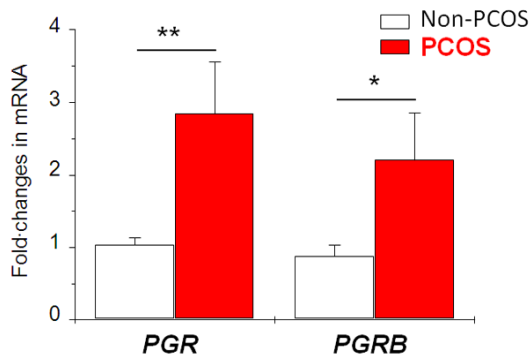


Figure 1. The expression of endometrial PGR isoforms in women with and without PCOS. Quantitative RT-PCR analysis of PGR and PGRB mRNA levels in the proliferative phase between non-PCOS ($n = 9$) and PCOS ($n = 6$) patients. mRNA levels were normalized to the average level of ACTB (β -actin) and CYC1 (cytochrome c isoform 1) in the same sample. Values are expressed as means \pm SEM. Statistical tests are described in the Materials and Methods. * $P < 0.05$; ** $P < 0.01$.

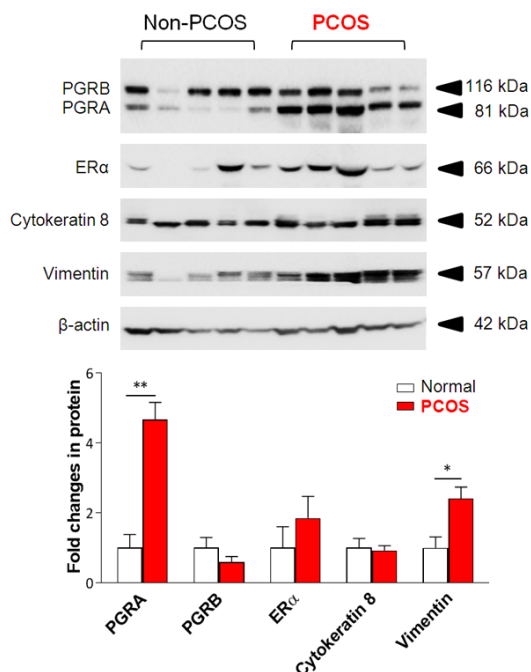


Figure 2. Comparison of the expression of endometrial PGR isoforms, ER α , and cellular marker proteins in women with and without PCOS. Western blot analysis of protein expression in the human endometrial tissues was performed. Representative images and quantification of the densitometric data for PGRA, PGRB, ER α , cytokeratin 8 (an epithelial cell marker), vimentin (a mesenchymal/stromal cell marker), and β -actin (a protein loading control marker) expression are shown ($n = 5$ /group). Values are expressed as means \pm SEM. Statistical tests are described in the Materials and Methods. * $P < 0.05$; ** $P < 0.01$.

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 (ER α , cytokeratin 8, vimentin, and Ki-67) were evaluated. As shown in **Figures 2** and **3**, although endometrial ER α protein level varied among individuals with no significant changes between non-PCOS and PCOS patients by Western blot analysis, a general increase in ER α 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 ER β in rat uterine stromal cells [36], and that treatment with estrogen induces PGR isoform expression in human endometrium

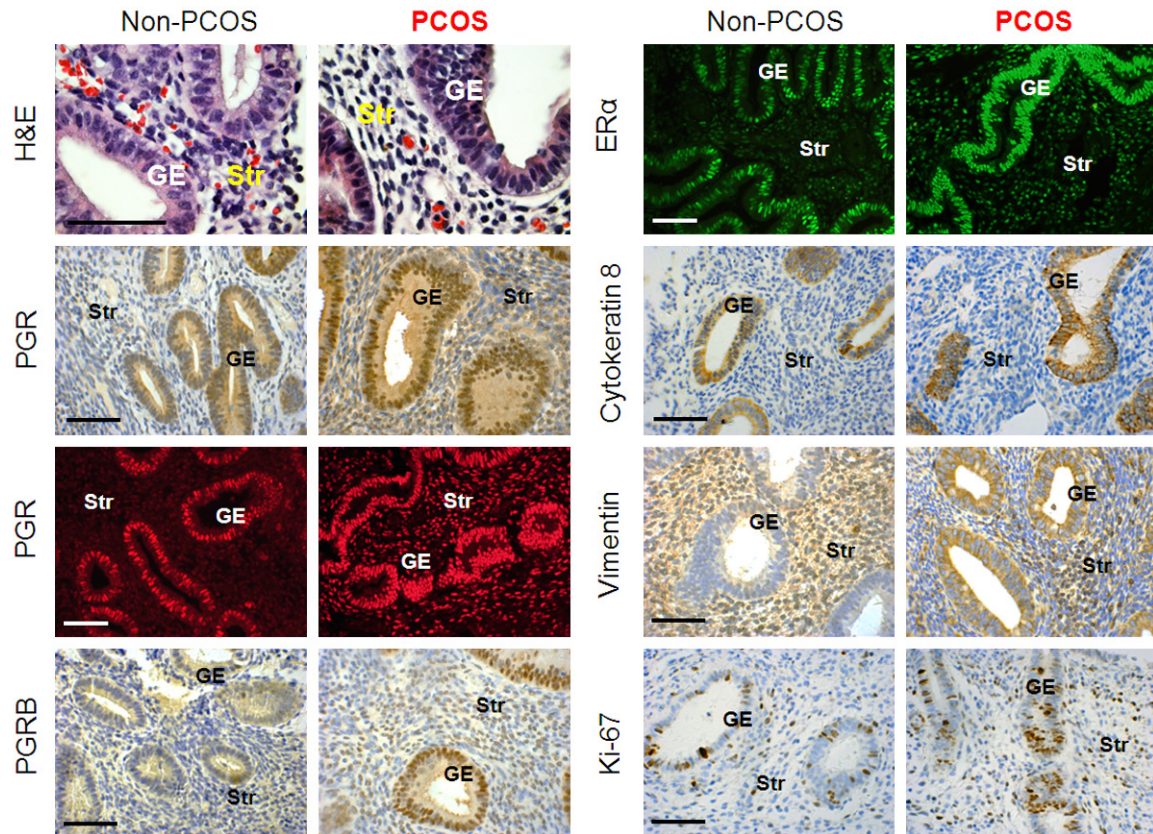


Figure 3. Comparison of the localization of endometrial PGR isoforms, ER α , and cellular marker proteins in women with and without PCOS. Immunohistochemical and immunofluorescent staining of PGR, PGRB, ER α , cytokeratin 8, vimentin, and Ki-67 in the endometrial tissue sections from non-PCOS ($n = 5$) and PCOS ($n = 3$) patients. The tissue sections were counterstained with hematoxylin. Ge, glandular epithelial cells; Str, stromal cells. Scale bars (100 μ m) are indicated in the photomicrographs.

[37]. Our data show that increased PGR isoform expression is associated with elevated ER α 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 ER α / 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 TNF α in the peripheral circulation of PCOS patients [39, 40]. Importantly, P4 suppresses TNF α -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, TNF α , 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.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Linus R Shao, Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at The University of Gothenburg, Medicinargatan 11, P. O. Box 434, Göteborg 40530, Sweden. Tel: +46 31-7863408; Fax: +46 31-786-3512; E-mail: linus.r.shao@fysiologi.gu.se

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