Increased protein expression of LHCG receptor and 17α-hydroxylase/17-20-lyase in human polycystic ovaries

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STUDY QUESTION: Does the expression of LHCG receptor (LHCGR) protein and key enzymes in the androgen biosynthetic pathway differ in normal human versus polycystic ovarian tissue?

SUMMARY ANSWER: LHCGR and 17α-hydroxylase/17-20-lyase (CYP17A1) protein levels are increased in polycystic ovaries (PCOs).

WHAT IS KNOWN ALREADY: The predominant source of excess androgen secretion in women with polycystic ovary syndrome (PCOS) is ovarian theca cells but few studies have directly assessed the presence and abundance of protein for key molecules involved in androgen production by theca, including LHCGR and the rate-limiting enzyme in androgen production, CYP17A1.

STUDY DESIGN, SIZE, DURATION: This is a laboratory-based, cross-sectional study comparing protein expression of key molecules in the androgen biosynthetic pathway in archived ovarian tissue from women with normal ovaries (n = 10) with those with PCOs (n = 16).

PARTICIPANTS/MATERIALS, SETTING, METHODS: A quantitative morphometric study was performed using sections of archived human ovaries (n = 26) previously characterized as normal or polycystic. The distribution and abundance of LHCGR, CYP17A1, 3β-hydroxysteroid dehydrogenase type 2 (3βHSDII) and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) proteins were evaluated by immunohistochemistry and quantified.

MAIN RESULTS AND THE ROLE OF CHANCE: A higher proportion of theca cells from anovulatory PCO expressed LHCGR protein when compared with control ovaries (P = 0.01). A significant increase in the intensity of immunostaining for CYP17A1 was identified in antral follicles in sections of PCO compared with ovaries from normal women (P = 0.04).

LIMITATIONS, REASONS FOR CAUTION: As the study used formalin-fixed ovarian tissue sections, it was not possible to carry out studies ‘in vitro’ using the same ovarian tissues in order to also demonstrate increased functional activity of LHCGR and CYP17A1.

WIDER IMPLICATIONS OF THE FINDINGS: The data are in keeping with the results of previous studies in isolated theca cells and support the notion of an intrinsic abnormality of theca cell androgen production in women with PCOS.

STUDY FUNDING/COMPETING INTEREST(S): The research was supported by a Programme Grant, G0802782, from the Medical Research Council (MRC) UK and by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. F.V.C was supported by Capes Foundation (Brazilian Ministry of Education). The authors have no conflicts of interest to disclose.

Key words: polycystic ovary syndrome / ovarian steroidogenesis / LHCG receptor / 17α-hydroxylase/17-20-lyase / theca cells

Introduction

Androgen excess is the biochemical hallmark of polycystic ovary syndrome (PCOS). It is estimated that upwards of 60% of women with PCOS have clinical manifestations of hyperandrogenism (acne, alopecia, hirsutism) and in 60–80% of women there is biochemical evidence of increased testosterone in the serum (Conway et al., 1989; Balen et al., 1995; Diamanti-Kandarakis et al., 1999; Chang et al., 2005). The higher circulating concentration of androgens in PCOS mainly reflects excessive production from the ovarian theca cell compartment, although in approximately one-quarter of women with PCOS an adrenal source for androgen excess co-exists (Kumar et al., 2005; Carmina and Lobo, 2007).
Theca cells from polycystic ovaries (PCOs) have an increased capacity to synthesize androgens both in primary culture (Gilling-Smith et al., 1994) and after several passages (Nelson et al., 1999). LH is the major endocrine factor driving secretion of androgens by theca cells and these cells have a higher expression of mRNA for LHCG receptor (LHCG), compared with control theca, as well as over-expression of steroidogenic factors, such as steroidogenic acute regulatory protein (STAR), cholesterol side-chain cleavage (CYP11A1) and, most importantly, as a key enzyme in androgen synthesis, 17α-hydroxylase/17,20-lyase (CYP17A1) (Gilling-Smith et al., 1997; Jakimiuk et al., 2001; Wickenheiser et al., 2005). In particular, greater stability and extended half-life of mRNA for CYP17A1 due to a post-transcriptional modification in CYP17A1 expression have been described (Nelson et al., 1999).

All these studies point to an intrinsic abnormality of theca cell function in PCOS but, to our knowledge, there are no studies which have comprehensively compared protein expression of both LHCGR and steroidogenic enzymes in polycystic and normal ovaries. Previous immunohistochemical studies have described localization of CYP17A1 and 3β-hydroxysteroid dehydrogenase type 2 (3βHSDII) protein at different stages of follicle development in sections of PCO but the numbers of ovaries and follicles in these studies have been small and none has reported on expression of LHCG or 17β-hydroxysteroid dehydrogenase type 5 (17βHSD type 5) (Tamura et al., 1992; Takayama et al., 1996; Kaajik et al., 2000).

Therefore, the main objective of this study was to describe in detail and quantify the distribution and intensity of protein expression for LHCGR and key steroidogenic enzymes (CYP17A1, 3βHSDII, 17βHSD type 5) in sections of normal ovaries and PCO, using an archive of well-characterized histological samples. Using this archive, we were also able to further characterize the expression of these proteins in women with PCO according to whether tissues were obtained from women with regular menstrual cycles or oligo/amenorrhoea.

**Materials and Methods**

**Sample collection**

Sections of archived human ovaries (formalin-fixed paraffin-embedded) removed (for benign gynecological disease) from patients aged <50 years were obtained from the Histopathology Bank of St. Mary’s Hospital (Imperial College Healthcare NHS Trust) London. Three groups were defined: normal women, characterized by a history of regular menses, normal ovarian morphology and evidence of ovulation (corpus luteum and/or corpus albicans); anovulatory PCO, defined by PCO with evidence of ovulation and anovulatory PCO, identified by the presence of PCO without evidence of ovulation, as described in detail in previous studies (Stubbs et al., 2005, 2007). Overall, sections from 26 human ovaries were obtained from women with ovulatory PCOs (n = 11), anovulatory PCOS (n = 5) and normal women, without PCOS (n = 10).

**Morphological classification**

The classification of follicle stages from small pre-antral to large antral follicles was made as previously described in the literature (Stubbs et al., 2005, 2007). The focus in this study was on growing follicles comprising more than one layer of granulosa cells (GCs), i.e. from the secondary stage onwards, which is the earliest stage at which a distinctive theca layer can be observed. Atresia in antral follicles was assessed according to the classification of Gougeon (1996).

**Immunohistochemistry**

The expression of LHCGR, CYP17A1, 3βHSDII and 17βHSDS was determined by immunohistochemistry in paraffin-embedded sections. The specific primary antibodies used were a mouse monoclonal antibody against the rat LHCGR (P1B4, a gift from Dr J Wimalasena, Knoxville, TN, USA), a mouse monoclonal antibody raised against CYP17A1 (kindly donated by Dr R Parker Jr., University of Alabama at Birmingham, USA), a rabbit polyclonal antibody against human 3βHSDII (a gift from Prof J Mason, University of Edinburgh, UK) and a rabbit polyclonal antibody to human AKR1C3 (17βHSD type 5) obtained from Abcam (Cambridge, UK).

Sections were processed as previously described (Stubbs et al., 2005, 2007). After setting up antibody dilution curves to determine the optimal concentrations of antibodies, the final dilutions of antibody in 10% serum in phosphate-buffered saline were, respectively, LHCGR, 1:4000; CYP17A1, 1:40; 3βHSDII, 1:200; 17βHSD type 5, 1:200 (Dharia et al., 2004; Papacleovoulou et al., 2009). The immunohistochemistry protocol for LHCGR expression in sections of human ovary was a modification of that previously described in studies of tissues in non-primate animal models (Teers and Dorrington, 1995; Peters et al., 2001; Schoemaker et al., 2002). Secondary antibodies chosen were goat anti-mouse BA9200 (Vector, Peterborough, UK) for LHCGR or CYP17A1, and goat anti-rabbit E0432 (DAKO, Denmark) for the other proteins. Both secondary antibodies were used at a dilution of 1:200 and incubated for 60 min at room temperature followed by processing using a peroxidase-conjugated avidin–biotin complex (RTU-ABC Kit; Vector). Finally, tissue sections were exposed to horse-radish peroxidase-conjugated 3,3′-diaminobenzidine tetrahydrochloride to permit visualization of labelling. Sections were counterstained with haematoxylin (BDH, Poole, UK), re-hydrated in alcohol (70, 95 and 100%) and mounted in DPX after two changes (5 min) in Histoclear®. Slides examined after replacement of the primary antibody by non-immune serum served as negative controls.

**Analysis of images**

Sections were examined using a Nikon E600 microscope (Nikon UK Ltd, Kingston-upon-Thames, UK) and images captured with a DXM 1200 digital camera (Nikon) with the assistance of Lucia software (image analysis programme, Nikon) or NIS-Elements-Ar (Nikon). Pictures were taken at the same microscope settings for each experiment and at 10, 20, 40 or 60 times magnification, in accordance with the requirements of each study. All images were saved in TIFF format. For each protein of interest, the areas for analysis in antral follicles were chosen using a programme to generate a random number sequence (1–8) corresponding to eight different zones, arbitrarily established (Graphpad Software, Inc., San Diego, CA, USA). Only healthy follicles were studied (unless otherwise mentioned) and staining for each protein was classified as present (positive) or absent (negative). However, it should be noted that where atretic follicles were described, this classification refers to the preponderance of pyknotic nuclei in GCs within the follicle and is not applicable to theca cells within the same follicle. In the case of CYP17A1, a semi-quantitative determination was used to assess the intensity of staining ranging from absent (−) to strong (+ +) staining. This method was complemented, as described in the Results section, by quantitative measurement of labelling, measuring density using the NIS-elements-Ar microscope imaging software (Nikon). Ovary sections from each patient were allocated an anonymous code and antral follicles were individually numbered. An area of the follicle was randomly selected and density of labelling was quantified. The density of labelling (an estimate of absorbance) in each theca cell was recorded and the mean of these individual values (in arbitrary units) was used for comparison between groups.

**Statistical analysis**

Where appropriate, data collected were stored in Excel (.xls) files. Statistical analysis was performed using Instat 3 software and the graphs were created.
and analysed using Prism6® software (both Graphpad). For each protein, differences between groups were analysed on the basis of number of ovaries (patients) per group and not on the total number of follicles or cells in order to take into account variation between subjects. As some of the data were not normally distributed, differences between groups of sections (i.e. differences between patients with and without PCOS) were tested using Student’s t-test (if normal distribution) or Mann–Whitney test (if asymmetrically distributed). For the comparison of more than two groups the Kruskal–Wallis test and an additional (Dunn’s multiple comparison) test were performed. In all cases, a probability value ($P \leq 0.05$) was considered as statistically significant. Normally distributed data were represented by mean ± SD. Skewed data were represented by median plus interquartile range (IQR).

Ethical approval
All specimens were anonymized and had been collected with informed consent and in conformity with the local Research Ethics Committee.

Results

**LHCGR protein in normal and PCO**

Specific immunostaining for LHCGR in ovary was detected in antral follicles (Fig. 1A and B) and confined to the cytoplasm of theca cells, and less frequently, of luteinized GCs (Fig. 1C). Overall, 96 follicles (25 from normal ovaries, 58 from ovulatory PCO and 13 from anovulatory PCO) were analysed. No differences were observed in terms of the proportion of cells staining for LH in follicles from ovari
ty women with PCO compared with normal women (Fig. 2A). However, a higher proportion of theca cells from anovulatory PCO expressed LHCGR when compared with controls ($P = 0.04$) (Fig. 2A). The same trend was found in the analysis of only healthy follicles (Fig. 2B) and also applied to the results of analysis of follicles in which GCs (but not theca cells) showed evidence of atresia ($P = 0.02$) (Fig. 2C).

**CYP17A1 protein in normal and PCO**

Immunoreactivity for CYP17A1 protein was identified in the cytoplasm of theca cells (internal and external layer) (Fig. 3A–D), theca–lutein and granulosa–lutein cells of the corpus luteum, and some dispersed interstitial cells in the ovarian stroma (not shown). Overall, 26 follicles (1206 theca cells) in normal ovaries and 58 follicles (3052 theca cells) from PCO were analysed (Table I).

The proportion of theca cells expressing CYP17A1 was similar between controls and PCO (Table I), but there was a clear increase in the intensity of staining in follicles from both PCO groups (data combined) compared with normal ovaries (Fig. 4A). The proportion of strong staining (+ +) for CYP17A1 in theca cells from PCO follicles was (mean, IQR) 0.34 (0.19–0.50) compared with 0.13 (0.04–0.25) in normal ovaries ($P = 0.01$) (Table I and Fig. 4A). This striking difference in labelling persisted in the subgroup of healthy follicles (Fig. 4B). The density of expression measured in optical density arbitrary units was also significantly increased in PCO theca cell layers in comparison with ovaries from normal women: the value in the PCO group was more than the double that observed in controls (Fig. 4D).

**3βHSDII Protein in the human ovary**

Immunolabelling for 3βHSDII protein was observed mainly in the cytoplasm of theca cells, theca–lutein cells and, faintly, in antral GCs (Fig. 3E). For this study, 9 follicles (274 theca cells) in normal ovaries and 60 follicles (1506 theca cells) in PCO ovaries were examined. As

![Figure 1](https://example.com/figure1.png)

**Figure 1** LHCG receptor (LHCGR) immunolabeling in human ovaries. Immuno-active LHCGR was present in theca cells in antral follicles (A and B). With the exception of luteinized GCs (white arrow) (C), no staining in GCs was observed either in pre-antral (D) or antral stages (A and B). (E) The negative control (first antibody replaced by normal mouse serum); a positive control (rat theca cells, black arrow) is shown in the small inset (F).
LHCG receptor protein expression in polycystic ovaries

Figure 2 Proportion of human theca cells expressing LHCG in anovulatory PCOs, ovulatory PCO and normal ovaries. Expression of LHCG was detected only in antral follicles in sections of normal ovaries (control, n = 5–7), and PCO from women with regular cycles (ovPCO, n = 7–9) or oligomenorrhea (anovPCO, n = 2–3). A significant increase in the proportion of theca cells expressing LHCG was identified in total follicles (A) (*P = 0.04) and atretic follicles (C) (**P = 0.02). The same increase was observed in healthy follicles from the PCO group (B), although this did not reach statistical significance. Graphs show median and IQR. Differences between groups were analysed on the basis of mean cell count per section (patient) and not on total number of follicles or cells.

I7βHSD Type 5 protein in the human ovary

Cells showing positive labelling for I7βHSD type 5 were identified in theca and GCs in antral follicles, and theca–lutein and granulosa–lutein cells in corpus luteum (Fig. 3F). Altogether, 23 follicles (1948 theca cells) in ovaries from normal women and 68 follicles (4860 theca cells) in PCO ovaries were analysed. As with 3βHSDII, there was similar expression of 17βHSD5 protein in theca cells from PCO and normal ovaries, either in total (Table I) or healthy follicles alone (mean ± SD 0.54 ± 0.20 versus 0.51 ± 0.28).

Discussion

The key findings of this study were the increased expression of both LHCGR and CYP17A1 protein in PCO compared with normal ovaries, emphasizing the importance of both factors in the etiology of androgen excess in PCOS. To our knowledge, this is the first study to compare protein expression of both LHCGR and key steroidogenic enzymes in ovarian tissue from women with and without PCOS. There were differences in the expression of LHCGR in theca cells in women with PCO and oligo/amenorrhea versus women with regular cycles, whether they had normal or PCO. These findings are reminiscent of our previous observations that anovulatory women with PCO show greater aberrations in pre-antral follicle development (Webber et al., 2003; Franks et al., 2008) and in the response of antral follicles to LH (Willis et al., 1996) than those with PCO and regular cycles. Similarly, the finding that the expression of CYP17A1 was increased in the theca of PCO in both ovulatory and anovulatory women is consistent with data showing increased androgen production from PCO theca cells in culture and in vivo, irrespective of cycle history (Gilling-Smith et al., 1994, 1997).

It has proved notoriously difficult to identify, reliably, LHCGR protein in the mammalian ovary by immunohistochemistry and we were fortunate in this study to be able to use an antibody that not only shows high sensitivity but also demonstrates clear specificity for LHCGR (Teerds and Dorrington, 1995; Peters et al., 2001; Schoemaker et al., 2002). This was a monoclonal antibody, a gift from Dr J Wimalasena (University of Tennesee), raised against purified LH receptors, which was shown to detect specific LHCGR immunoactivity within ovarian theca cells (and Leydig cells of the testis) in various species. We are aware of no other studies to date, particularly in women rather than animal models, that have used these methods to address the role of LHCGR in the pathogenesis of PCOS. In normal ovaries, LHCGR was identified in theca–lutein cells and external and internal theca, as expected from studies in experimental animals (Teerds and Dorrington, 1995; Peters et al., 2001; Schoemaker et al., 2002). The finding that a higher proportion of theca cells in tissue from anovulatory women with PCOS expressed LHCGR protein than in normal ovaries is in accordance with previous reports of increased mRNA content of LHCGR in theca cells from PCOS ovaries (Nelson et al., 1999) and may be an important contributory factor in the mechanism underlying enhanced androgen synthesis in PCO. This observation is reflected in a study of a sheep model of PCOS; Hogg et al. (2012) found that LHCGR gene expression (as well as the expression of CYP17A1) was increased in theca cells from the prenatally androgenized sheep. In another animal model of PCOS, Zhu et al. (2010) observed hypo-methylation of the LH receptor gene in the dehydroepiandrosterone-induced rat. These data support...
the view that exposure to excess androgen during development or in post-natal life may affect both LH receptor function and steroidogenic enzyme activity. LH is the major extra-ovarian factor driving secretion of androgens by theca cells (Wickenheisser et al., 2006; Diamanti-Kandarakis, 2008; Young and McNeilly, 2010). Importantly, recent data from large-scale genetic association studies of women with PCOS have pointed to susceptibility loci in Chromosome 2 close to the genes encoding gonadotrophin receptors, suggesting that there may be a genetic component to LHCGR dysfunction in PCOS (Chen et al., 2011; Shi et al., 2012; Mutharasu et al., 2013).

The microsomal enzyme CYP17A1 is a major regulatory enzyme in steroid production in the gonads and adrenal glands, and a key step in androgen production. In this work, about one-third of theca cells of PCO (nearly twice the proportion in normal ovaries) strongly expressed CYP17A1 (Fig. 4A). This substantial difference was also evident when a more formal quantification of labelling was made using measurement of optical density (Fig. 4D). Therefore, the increase in the presence of CYP17A1 protein in theca cells observed in this investigation, in conjunction with other data in the literature showing hypersecretion of androstenedione and augmented transcription, greater mRNA content and stability of CYP17A1 gene, support a major action for CYP17A1 as an enhancer of androgen secretion in PCOS (Wickenheisser et al., 2006).

The other steroidogenic enzymes that were evaluated in this study were 3β-HSDII and 17β-HSD type 5, which are both significant participants in the androgen synthesis cascade. 3β-HSDII is an activating enzyme which is essential for the conversion of dehydroepiandrosterone to androstenedione; the second (17β-HSD type 5) converts androstenedione to the much more potent androgen, testosterone (Bremer and Miller, 2008). In contrast to the findings with regard to CYP17A1, we found no differences in the distribution or the intensity of expression of protein for either 3β-HSDII or 17β-HSD type 5, suggesting that altered regulation of these enzymes does not contribute significantly to androgen excess in PCOS.

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<tr>
<th>CYP17A1</th>
<th>3β-HSD II</th>
<th>17β-HSD type 5</th>
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<tr>
<td><strong>Normal (n = 9)</strong> (26 follicles)</td>
<td><strong>PCO (n = 14)</strong> (58 follicles)</td>
<td><strong>PCO (n = 14)</strong> (58 follicles)</td>
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<tr>
<td>Total staining</td>
<td>Strong staining</td>
<td>Total staining</td>
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<tr>
<td><strong>(0.45 – 0.78)</strong></td>
<td><strong>(0.04 – 0.25)</strong></td>
<td><strong>(0.58 – 0.87)</strong></td>
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Data show the proportion of steroidogenic cells with positive staining for the proteins, expressed as median and IQR ranges. The number of ovaries per group (n) and the number of follicles analysed are indicated. CYP17A1, 17α-hydroxylase/17-20-lyase; 3β-HSD II, 3β-hydroxysteroid dehydrogenase type 2; 17β-HSD type 5; 17β-hydroxysteroid dehydrogenase type 5.

* A significant difference (P < 0.01) in the proportion of theca cells with strong expression of CYP17A1 in normal versus PCO.

Figure 3 CYP17A1, 3β-HSDII and 17β-HSD type 5 immunolabelling in human ovaries. Specific staining for 17α-hydroxylase/17-20-lyase (CYP17A1) was observed in theca cells in early antral follicles (A), and in healthy (B), luteal (C) and atretic (D) antral follicles. Labelling for CYP17A1 was present in the theca interna (B, black arrow) and theca externa (B, white arrow). Staining for 3β-hydroxysteroid dehydrogenase type 2 (3β-HSDII) (E) or for 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD type 5) (F) was also identified in antral follicles. Negative controls are displayed in the insets for CYP17A1 (B), 3β-HSDII (E) and 17β-HSD type 5 (F).
An important strength of the findings in the current study is that these were obtained from a very well-characterized archive of ovarian tissue, which has been used previously in studies aimed at defining which molecules are involved in abnormalities in follicle development in PCOS. A limitation of our study is that, because we used archived, fixed and paraffin-embedded ovarian tissue, we were not able to examine gene expression by way of mRNA levels or to quantify protein concentrations (by western immuno-blotting) or enzyme activity. In conclusion, our results from histological sections provide robust evidence for a significant disruption (i.e. increase) of LHCGR and CYP17A1 protein expression in PCO tissue, which support the findings of previous studies based on gene expression in isolated theca cells. These observations are in keeping with the notion that there are intrinsic aberrations of ovarian function in PCOS, which range from disorders of follicle formation and activation (Webber et al., 2003; Stubbs et al., 2005, 2007; Franks et al., 2008) to disturbances in gonadotrophin action and steroidogenesis. The data from animal models suggest that exposure to excess androgen during ovarian development induces or, at least enhances, the expression of CYP17A1 but it remains unclear whether aberrant steroidogenesis in the human PCO is the cause or consequence of disordered early follicle development.

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Authors’ roles

F.V.C. was responsible for the laboratory work and drafting the manuscript. K.H. and S.F. conceived and supervised the study. K.T. supplied the expertise and materials for the LHCGR immunohistochemistry, and with K.H. and S.F. analysed, organized the data and made major contributions to the writing of the paper.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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