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PCOS

Granulosa cell aromatase enzyme activity: Effects of follicular fluid from patients with polycystic ovary syndrome, using aromatase conversion and $[^{11}C]$vorozole-binding assays

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Abstract

The local regulation of ovarian aromatase enzyme in polycystic ovary syndrome (PCOS) was studied with aromatase conversion and $[^{11}C]$vorozole-binding assays to analyze aromatase activity, substrate–enzyme affinity and number of aromatase binding sites in non-cultured human granulosa cells (GC) incubated with different sources and preparations of follicular fluid (FF). Incubation with FF from women stimulated in in vitro fertilization cycles with follicle-stimulating hormone yielded higher conversion activity than with FF from healthy women and PCOS patients, paralleled with higher substrate affinity (lower $K_d$) than with FF from healthy women. In PCOS women, charcoal-pretreated FF yielded higher conversion, whereas the ether-pretreated FF yielded lower conversion activity, than with untreated PCOS FF. Both preparations of FF yielded higher affinity to substrate (lower $K_d$ values) and the ether-pretreated FF a lower number of binding sites ($B_{max}$). It seems that steroids with the presence of proteins in PCOS FF reduced aromatase conversion activity through decreased substrate affinity, whereas FF preparations devoid of proteins reduced the aromatase conversion activity mainly through blocking of aromatase active sites. Identification of specific agents responsible for this rapid regulation of aromatase function might help to understand normal regulation of the menstrual cycle and supposed imbalances of inhibitors/activators in PCOS.

Keywords: Aromatase, inhibition, follicular fluid, polycystic ovary syndrome, $[^{11}C]$vorozole

Introduction

It is generally accepted that estrogen is needed for development of the selected follicle. The capacity of the dominant follicle to synthesize estrogens increases dramatically as the follicle transits from early follicular to preovulatory phase [1,2]. Selection of the dominant follicle in the early follicular phase is under the control of gonadotropins [3–5]. However, there is evidence suggesting that the activity of aromatase, the enzyme synthesizing estrogens, can be regulated locally by compounds in the follicular fluid (FF), as shown in pigs [6] and humans [7–9].

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disorder characterized by hyperandrogenism and anovulatory infertility [10]. In PCOS, follicular development arrests at the stage of selection of the dominant follicle when aromatase activity in the granulosa cells (GC) and the production of estradiol ($E_2$) would normally increase [2]. The exact mechanism by which follicle selection is blocked in PCOS is not known. Concentrations of...
folicular-stimulating hormone (FSH) and of the aromatization substrate (androstenedione) in FF from PCOS patients are sufficient to support maximal rates of E₂ synthesis [11] and GC from PCOS patients respond readily to FSH stimulation in vitro [12]. A hypothesis has been proposed postulating that PCOS FF contains an inhibitor of E₂ biosynthesis [13].

Positron emission tomography (PET), using short-lived radionuclides (¹¹C, ¹⁸F and ¹⁵O), can follow biological processes such as blood flow, utilization of glucose, oxygen and amino acids, and receptor binding, in vivo as well as in vitro. Vorozole is a high-affinity non-steroidal competitive aromatase inhibitor [14] developed for the purpose of inhibiting the aromatization of androgens to estrogens in patients with hormone-dependent breast cancer [15]. Vorozole can be labeled with ¹¹C [16]. Quantification of aromatase in GC is based on binding of [¹¹C]vorozole to the active pocket of aromatase enzyme. The results of our previous studies suggest that an in vitro technique including [¹¹C]vorozole binding can be used for aromatase quantification with very high sensitivity [17].

In the present study, the effects of FF from PCOS women, as well as its fractions devoid of steroids (charcoal-pretreated PCOS FF) and devoid of proteins (ether-pretreated PCOS FF), on [¹¹C]vorozole binding and GC androgen conversion activity were measured and compared with those of FF from endocrinologically healthy women and from in vitro fertilization (IVF) patients undergoing FSH stimulation, to elucidate mechanisms and differences in the local regulation of GC aromatase enzyme activity in PCOS women and healthy controls.

Methods

Clinical material

The study subjects were recruited by advertising in the media and investigated at the Donetsk Regional Center of Mother and Child Care, Donetsk, Ukraine, which functions as a university clinic. Forty PCOS patients (PCOS group) and 46 normally cycling women (control group) were recruited after oral and written information. The diagnosis of PCOS was based on amenorrhea or oligomenorrhea (<10 cycles per year), a characteristic ovarian image on ultrasound examination (≥10 small follicles per plane, in association with a marked ovarian stroma) [18], hirsutism (which was assessed by a modified version of the protocol used by Ferriman and Gallwey [19]; a woman with a score of ≥8 was considered clinically hirsute) and hyperandrogenemia. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). All ultrasound examinations were performed transabdominally or transvaginally (3.5 and 5 MHz sector probe, respectively; Kranzbühler GmbH, Solingen, Germany). The PCOS patients were treated for infertility by ovarian wedge resection.

Control subjects were women with infertility presumably caused by pelvic adhesions. They had regular cycles and normal ovaries on pelvic ultrasound examination. All subjects were in good general health and had not taken hormonal medication or oral contraceptives during the three months before inclusion in the study. Ultrasound images from PCOS women and controls made in Ukraine were blindly evaluated by two independent Swedish gynecological ultrasound experts.

In addition, FF was sampled from seven Swedish patients attending IVF treatment because of male-factor, tubal-factor, non-ovarian endometriosis or unexplained infertility. These patients attended infertility treatment in Uppsala University Hospital and Carl von Linne IVF clinics, Uppsala, Sweden. The treatment protocol of IVF patients consisted of pituitary downregulation by gonadotropin-releasing hormone analog (buserelin; Hoechst, Frankfurt, Germany) employing the ‘long’ protocol initiated at the mid-luteal phase (900 µg/day, intranasal administration). Recombinant FSH (Gonal-F®; Serono SA, Aubonne, Switzerland) (subcutaneous) was injected daily (225 IU/day) starting on cycle day 3. Dose adjustment was performed, when necessary, from cycle day 7. Human chorionic gonadotropin (hCG) (Profasi®; HP; Serono SA), 10 000 IU, was administered when one or more follicles reached a maximal diameter >23 mm, with E₂ concentrations corresponding to the number of follicles. Transvaginal oocyte retrieval was performed under ultrasound guidance 36 h after hCG administration. Follicles larger than 15 mm in diameter were aspirated.

Ethical considerations

Informed consent was obtained from all women, and the study protocol was approved by the Human Ethics Committee of the Medical Faculty of Uppsala University (Upps d:nr 99 167; 1999-04-14) and by the Ethics Committee of the Research Institute of Family Medical Problems, Donetsk State Medical University (1999-01-04).

Collection of follicular fluids

FF from PCOS and healthy follicles of 5–8 mm diameter was pooled within each subject and centrifuged. Size of the follicles was measured by transvaginal ultrasonography performed during laparoscopic treatment of PCOS (wedge resection) or adhesiolsis (controls). Sampling was performed between days 3 and 7 in the follicular phase in regularly menstruating women and at any day in oligo-/amenorrheic patients. The samples were kept...
at −20°C until analysis. The frozen FF samples were packed in dry ice during the transportation from Donetsk to Uppsala. Addition of FF from FSH-stimulated Swedish IVF patients and GC donors resulted in three main study groups: PCOS, control women and FSH-stimulated women (stimulation protocol described in ‘Clinical material’).

Treatment of follicular fluids before experiments in vitro

Thawed FF from three to five controls, two or three PCOS patients and fresh FF obtained from Swedish IVF patients – donors of GC – were used to prepare five separate pools (for every set of experiments a new pool was prepared): (1) FF from control women, (2) untreated PCOS FF, (3) charcoal-pretreated PCOS FF, (4) ether extract from PCOS FF and (5) FSH-stimulated FF. The control incubate was Dulbecco’s FF, (4) ether extract from PCOS FF, (5) FSH-untreated PCOS FF, (3) charcoal-pretreated PCOS pool was prepared: (1) FF from control women, (2) FF from FSH-stimulated women (stimulation protocol described in ‘Clinical material’).

Assay of aromatase activity (estrogen synthesis)

Aromatase activity was assessed by measuring the [3H]2O produced by stereospecific release of 3H from the C-1β position of 4-androstene-3,17-dione ([1β-3H]-A4), as previously described [20]. In brief, 10 μl of an ethanol solution containing [1β-3H]-A4 (1 μCi, 40 pmol; New England Nuclear, Boston, MA, USA) was added to 0.1 ml of FF containing 15 000 GC. Incubation was performed in duplicate together with boiled blanks. The aromatase-catalyzed reaction was initiated by the addition of 100 μg NADPH (Sigma Chemical Co., St. Louis, MO, USA) in 50 μl of PBS, and GC were incubated for 2 h at 37°C. At the end of the reaction period, 0.5 ml of PBS was added to compensate evaporated liquid and steroids were extracted with three 5-ml portions of diethyl ether, which were discarded. Trichloroacetic acid (1.0 ml, 30%) was added to the remaining aqueous phase and the mixture was shaken. The precipitated proteins were sedimented by centrifugation for 10 min at 1500g, and 1 ml of the resulting supernatant was collected. Remaining steroids were removed by adding 1 ml of a suspension containing 0.5% (w/v) dextran and 5% (w/v) charcoal in PBS. After mixing and centrifugation for 20 min at 2000g, 1.0 ml of the clear supernatant was taken for liquid scintillation counting of [3H]2O radioactivity. Correction for blank values was performed and aromatase activity was expressed in terms of attomol estrogen formed/1000 cells/h. For presentation of results from the conversion assay and [11C]vorozole-binding assay, see below (‘Statistical analyses’).

[11C]Vorozole-binding assay

Each determination was performed in 15 000 GC in 0.08 ml of FF in different sets of experiments. [11C]Vorozole [16] ([N-methyl-11C]vorozole; 4.2 ± 2.0 Ci/μmol, range 2.1–7.0 Ci/μmol, >98% radiochemically pure) was added to yield vorozole concentration of 0.3, 1.0 and 5.0 nM. Duplicate samples were co-incubated with 1 μM non-radioactive vorozole for the assessment of non-specific binding. Six duplicate incubations were performed in different five different FF pools and DMEM. After incubation for 30 min, the cells were harvested and washed using a 48-sample cell harvester system (Brandel,
Gaithersburg, MD, USA). All incubation wells were simultaneously aspirated and the contents were passed through Whatman GF/B glass fiber filters (Brandel). Three sequential washings were performed with PBS. The filters were removed and placed on a storage phosphor plate (Molecular Dynamics, Sunnyvale, CA, USA) for 40 min exposure of $^{11}$C radioactivity. As a standard, a 20 µl sample of the 1 nM $[^{11}C]$vorozole incubation solution was deposited on a filter and exposed together with the GC samples.

The imaging plate was read with a laser phosphor imager (model 400S; Molecular Dynamics) and the digital image was stored for further quantitative determinations using the software ImageQuant (Molecular Dynamics) [21]. On the images, radioactivity regions were outlined to indicate the total radioactivity on each of the filters. The procedure included an automatic background subtraction. Quantification of the specifically bound radioactivity was performed by subtraction of each non-specific (1 µM vorozole) sample from its respective pair (1 nM). The total counts read from the 20 µl standard allowed calculation of a calibration factor expressed as fmol/count. Using this calibration factor, the specifically bound radioactivity was recalculated to be expressed in fmol.

Only five of seven binding experiments were technically successful, thus sample size of the binding study was $n = 5$.

**Statistical analyses**

Values of conversion assay and $[^{11}C]$vorozole binding were analyzed and presented as percentage of estrogen produced and radioactivity bound in comparison with that reached in the reference incubate (set as 100%). This was done to reduce the variation in the final results because of biological (different pools of FF and GC used for different runnings) and technical (potentially different concentrations of $[^{11}C]$vorozole used for different runnings) reasons, as well as for simple comparison of presented results. To estimate the influence of different incubates on aromatase conversion and $[^{11}C]$vorozole-binding assays, the mean values of the two methods, for each incubate separately, were compared using analysis of variance (ANOVA).

Differences between PCOS and control patients in age and BMI were assessed by ANOVA (normally distributed variables); in hirsutism index by the Wilcoxon two-sample test (non-normally distributed variable); in smoking habits and number of cycles during the past 12 months (categorical variables) by an appropriate $\chi^2$ test. Correlations between aromatase conversion activity and $B_{\text{max}}$ and $K_d$ of $[^{11}C]$vorozole binding, after adjustment for $K_d$ and $B_{\text{max}}$ respectively, were tested by Spearman’s partial correlation.

For every statistically significant result cited, the experiment-wise $p$ value is less than 0.05 unless otherwise specified. The statistical program JMP and SAS package (SAS Institute Inc., Cary, NC, USA) were used for statistical analyses.

**Results**

**Matching of patients with polycystic ovary syndrome and controls**

Control and PCOS groups were similar with regard to BMI and smoking habits but differed according to age (mean ± standard error of the mean (SEM): 27.63 ± 3.43 vs. 24.75 ± 3.42 years, respectively) ($p < 0.0001$), hirsutism index (median [range]: 2.83 [0–8] vs. 9 (4–24), respectively) ($p < 0.0001$) and number of cycles during the past 12 months (12/12 vs. 5.8/12) ($p < 0.0001$).

**Androgen conversion activity (estrogen production) and $[^{11}C]$vorozole binding in granulosa cells incubated with follicular fluid from defined groups of patients and sub-fractions of follicular fluid from patients with polycystic ovary syndrome**

Comparison with incubation of granulosa cells with Dulbecco’s modified Eagle’s medium. As shown in Table I, incubation of GC with FF from healthy women and PCOS patients both yielded significantly lower androgen conversion activity (estrogen production) (both $p < 0.001$) compared with incubation with DMEM, whereas FF from FSH-stimulated women yielded significantly higher activity ($p < 0.01$). In parallel, incubation with FF from healthy women and from PCOS women led to significantly lower affinity to substrate, with substantially (four- to seven-fold) and significantly higher $K_d$ of $[^{11}C]$vorozole binding (both $p < 0.05$). No significant differences were found in mean values of $[^{11}C]$vorozole maximal binding ($B_{\text{max}}$).

Spearman’s partial correlation showed that the overall variation of aromatase activity in healthy FF, PCOS FF and FSH-stimulated FF, as compared with that in DMEM, might be explained both by variation in the number of aromatase active sites available for conversion ($r_S = 0.56; p = 0.03$) and changes in affinity of the substrate to the enzyme ($K_d$) ($r_S = 0.67; p = 0.05$).

**Comparison with incubation of granulosa cells with follicular fluid from healthy women.** As shown in Table I, androgen conversion activity (estrogen production) was significantly higher in GC incubated with FF from FSH-stimulated women, as compared with incubation with FF from healthy women and PCOS patients (both $p < 0.001$), whereas the activity did not differ significantly between incubations with
Table I. Aromatase activity, dissociation constant ($K_d$) and maximal binding ($B_{max}$) of $[^{11}C]$vorozole binding in granulosa cells after incubation in follicular fluid from endocrinologically healthy women (Healthy), patients with polycystic ovary syndrome (PCOS) and women stimulated in in vitro fertilization cycles with follicle-stimulating hormone (FSH-stim), expressed as a percentage of means from incubation with Dulbecco’s modified Eagle’s medium (DMEM) (set at 100%). Values expressed as mean ± standard error of the mean.

<table>
<thead>
<tr>
<th>Aromatase assay</th>
<th>$[^{11}C]$Vorozole-binding assay</th>
<th>$K_d$</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{3}H]_2$O release</td>
<td>$[^{11}C]$Vorozole-binding assay</td>
<td>$K_d$</td>
<td>$B_{max}$</td>
</tr>
<tr>
<td>$n$</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DMEM</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Healthy</td>
<td>39 ± 8***</td>
<td>693 ± 242*</td>
<td>203 ± 61</td>
</tr>
<tr>
<td>PCOS</td>
<td>30 ± 8**,**†††</td>
<td>413 ± 127*</td>
<td>106 ± 16</td>
</tr>
<tr>
<td>FSH-stim</td>
<td>213 ± 52**,<strong>†††,</strong>†††</td>
<td>246 ± 64**</td>
<td>112 ± 18</td>
</tr>
</tbody>
</table>

Significance of difference from DMEM: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; i) significance of difference from Healthy: †††$p < 0.001$; ii) significance of difference between PCOS and FSH-stim: **$p < 0.01$, ***$p < 0.001$.

FF from PCOS and from healthy women. In parallel, the dissociation constant ($K_d$) of $[^{11}C]$vorozole binding was significantly lower (higher affinity) in GC incubated with FF from FSH-stimulated women ($p < 0.001$), whereas no significant difference in mean $K_d$ values was found between incubations with FF from PCOS women and from healthy women. No significant differences were found in $[^{11}C]$vorozole maximal binding ($B_{max}$) in GC between the three groups of FF.

Spearman’s partial correlation showed that the overall variation of aromatase activity in both charcoal-pretreated and ether-pretreated PCOS FF, as compared with that in untreated PCOS FF, was not independently explained by either a variation in affinity of the precursor to the enzyme ($r_S = -0.68$; $p = 0.044$) and less likely by changes in the number of aromatase active sites available for conversion ($r_S = 0.59$; $p = 0.10$).

Effects of follicular fluid from patients with polycystic ovary syndrome after pretreatment with charcoal (devoid of steroids) and its ether extract (devoid of proteins), compared with those of follicular fluid with no treatment. As shown in Table II, androgen conversion activity was significantly higher in GC incubated with charcoal-pretreated FF in contrast to significantly lower values after incubation with ether-pretreated PCOS FF (both $p < 0.0001$), compared with incubation with untreated FF from PCOS women. Conversion activity differed more than ten-fold between incubations with charcoal-pretreated and ether-pretreated PCOS FF ($p < 0.0001$). Affinity to substrate was significantly higher, as indicated by the $K_d$ of $[^{11}C]$vorozole binding being significantly lower, in GC incubated with both charcoal-pretreated and ether-pretreated PCOS FF, compared with that of untreated FF from PCOS women (both $p < 0.01$), whereas maximal $[^{11}C]$vorozole binding ($B_{max}$) was significantly lower for the ether-pretreated PCOS FF ($p < 0.001$). $K_d$ and $B_{max}$ mean values did not differ significantly between incubation with charcoal-pretreated FF and ether-pretreated FF from PCOS women.

Spearman’s partial correlation showed that the overall variation of aromatase activity in both charcoal-pretreated and ether-pretreated PCOS FF, as compared with that in untreated PCOS FF, was not independently explained by either a variation in affinity of the precursor to the enzyme ($r_S = -0.36$; $p = 0.34$) or changes in the number of aromatase active sites available for conversion ($r_S = 0.39$; $p = 0.30$).

Table II. Aromatase activity, dissociation constant ($K_d$) and maximal binding ($B_{max}$) of $[^{11}C]$vorozole binding in granulosa cells after incubation in follicular fluid from endocrinologically healthy women (Healthy), patients with polycystic ovary syndrome (PCOS FF) pretreated with charcoal (PCOS-charcoal) and ether extract of PCOS FF (PCOS-ether), expressed as a percentage of means from incubation with FF from PCOS patients (PCOS) (set at 100%). Values expressed as mean ± standard error of the mean.

<table>
<thead>
<tr>
<th>Aromatase assay</th>
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<th>$K_d$</th>
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</tr>
<tr>
<td>$n$</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>PCOS</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>PCOS-charcoal</td>
<td>421 ± 52***,**†††</td>
<td>40 ± 16**</td>
<td>74 ± 29</td>
</tr>
<tr>
<td>PCOS-ether</td>
<td>29 ± 4***,**†††</td>
<td>15 ± 2***</td>
<td>25 ± 8***</td>
</tr>
</tbody>
</table>

Significance of difference from PCOS: **$p < 0.01$, ***$p < 0.001$; significance of difference between PCOS-charcoal and PCOS-ether: †††$p < 0.001$.

Discussion
The main finding of the present study is that agents in FF can rapidly regulate GC aromatase enzyme function (estrogen synthesis) through changes in the substrate’s affinity to the enzyme and through blocking of the aromatase active site. Agents extracted by charcoal and ether pretreatment, respectively, seem to modify effects of each other. The present data indicate that agents in PCOS FF (most likely steroids) reduce GC aromatase conversion activity (estrogen synthesis) at least partly through reduced substrate affinity, because after charcoal pretreatment of PCOS FF conversion activity increased in parallel with increased affinity to substrate. The ether-pretreated PCOS FF (devoid of proteins but steroids present) further reduced conversion mainly through blocking the aromatase active site and despite further increased affinity. The increased conversion activity of FSH-stimulated FF seems to be mainly through an increased substrate affinity (reduced $K_d$). These results are based on comparison of the androgen conversion assay and a previously
developed and validated $[^{14}C]$vorozole-binding assay [17,22,23], with the assumption that the nature of binding of the aromatization precursor and $[^{11}C]$vorozole to the active site of the enzyme is similar.

It is important to underline that experiments aiming to elucidate the regulation of GC aromatase in follicles in vivo are preferably performed in vitro in physiological fluids (FF) instead of synthetic media, since the biological properties of these liquids are very different and the use of synthetic media might create artifacts.

Compared with incubation of GC with DMEM, incubation of GC with healthy FF and PCOS FF yielded substantially lower estrogen synthesis, whereas FSH-stimulated FF yielded higher estrogen synthesis. These results are in line with previous reports of inhibitory effects of unstimulated FF [8,12] and stimulating effects of FSH-stimulated human FF [8]. The magnitude of the inhibition observed in the present study was close to that reported previously [7,8]. The variation of aromatase conversion activity in GC incubated in PCOS and FSH-stimulated FF, as compared with that in healthy FF, might be explained by changes in affinity of the substrate to the enzyme in addition to changes in the number of available binding sites. This finding might modify and add to the generally accepted theory that stimulation of follicles with FSH might modify and add to the generally accepted theory that stimulation of follicles with FSH yields higher estrogen synthesis. These results are in line with previous reports of inhibitory effects of unstimulated FF [8,12] and stimulating effects of FSH-stimulated human FF [8]. The magnitude of the inhibition observed in the present study was close to that reported previously [7,8]. This finding might modify and add to the generally accepted theory that stimulation of follicles with FSH yields higher estrogen synthesis. These results are in line with previous reports of inhibitory effects of unstimulated FF [8,12] and stimulating effects of FSH-stimulated human FF [8].

Hillier and co-workers [3,25] hypothesized that a timely induction of the GC aromatase system and the attendant changes in the intrafollicular steroid profiles were major determinants for selection of the dominant follicle early in the ovarian cycle. Numerous compounds present in FF (activin, epidermal growth factor, interleukin-6, insulin-like growth factors and leukemia inhibitory factor, different steroids) [2] have the ability to negatively or positively modulate aromatase functional capacity. The approach selected in the present study used FF, a complex of all physiological factors (instead of synthetic media), to check its effect on aromatase properties (conversion activity and $[^{11}C]$vorozole binding) to avoid measurement of artifact which might appear when performing experiments in synthetic media. Presence of aromatase activator in the charcoal-pretreated PCOS FF preparation and presence of aromatase inhibitor in the ether-pretreated PCOS FF suggest that, in PCOS FF, the aromatase activating/inhibiting system is misbalanced towards blockage of the enzyme function. Thus, elucidation of the reasons for this misbalance might create a new hypothesis for the treatment of PCOS.

The results of the present study indicate that agents in FF from different types of patients differ in their effect on rapid changes in functional properties of ovarian aromatase enzyme. In the present study, PCOS FF seemed to reduce aromatase activity mainly by a reduction in affinity to substrate, an effect that was removed after pretreatment with charcoal; thus most likely these agents are steroids. The ether-pretreated PCOS FF, devoid of proteins but with steroids present, further reduced the conversion activity mainly through blockage of aromatase active sites and despite high affinity, and thus seemed to modify the effects of agents present in the charcoal extract (steroids). We hypothesize that these molecules are of protein character. Further elucidation of mechanisms and specific compounds responsible for rapid activation/inhibition of aromatase activity in ovarian follicles might be the next step to understand selection of the dominant follicle in
normal menstrual cycles and the anovulation in PCOS patients.

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