The mechanism of action of epidermal growth factor and transforming growth factor α on aromatase activity in granulosa cells from polycystic ovaries*

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We investigated aromatization and the mechanism of action of epidermal growth factor (EGF) and transforming growth factor α (TGFα) on oestradiol biosynthesis in freshly prepared granulosa cells from polycystic ovaries. Freshly prepared granulosa cells from polycystic ovaries incubated for only 3 h under basal conditions secreted significantly (P < 0.001) greater amounts of oestradiol-17β than that of granulosa cells from normal ovaries. 8-Bromo-cyclic adenosine monophosphate (8-Br-cAMP), but not follicle stimulating hormone (FSH) or luteinizing hormone (LH), further enhanced this activity. Both EGF and TGFα inhibited gonadotrophin- or 8-Br-cAMP-stimulated, but not basal, oestradiol production. LH receptor (LHR) binding, estimated by immunolabelling the bound LH, was significantly (P < 0.001) reduced in granulosa cells from polycystic ovaries when compared with cells from normal ovaries. EGF or TGFα significantly reduced the binding in cultured cells from all patient groups (P < 0.05). More interestingly, a further increase of the inhibitory effect was seen in granulosa cells from polycystic ovaries (P < 0.001). In conclusion, granulosa cells from polycystic ovaries contain high levels of basal aromatase activity in vitro, which is probably inherited from the in-vivo condition. EGF and TGFα suppress oestradiol synthesis at a step beyond the production of cAMP and also LHR binding with more effect in granulosa cells from polycystic ovaries.

Key words: aromatase/gonadotrophins/growth factors/PCOS

Introduction

Polycystic ovarian syndrome (PCOS), a common cause of anovulatory infertility, is a heterogenous and complex disease with unknown aetiology. This syndrome is characterized by a sharp reduction in ovarian cyclic oestradiol production (Erickson et al., 1992; Mason et al., 1994). However, ovaries of patients with PCOS frequently respond to exogenous gonadotrophin administration by the excessive production of oestrogen and an increased risk of hyperstimulation syndrome (Armar et al., 1990). Moreover, dispersed granulosa cells from untreated polycystic ovaries (PCO) produce significantly more oestradiol in cultures than granulosa cells from normal ovaries (Haney et al., 1986; Almahbobi et al., 1996). These data suggest that aromatase is functional and highly active in PCO, but only after gonadotrophin stimulation and/or long-term granulosa cell culture in vitro. These apparent inconsistencies in aromatase activity in PCOS, in vivo and in vitro, raise questions regarding the control of the expression and activity of aromatase.

In previous reports, it has been suggested that PCOS is likely to be a result of an exaggerated action of intraovarian regulator(s) exerting dual effects, both stimulatory and inhibitory (Fauser 1994; Almahbobi and Trounson, 1996). Increasing evidence suggests that epidermal growth factor (EGF) and transforming growth factor α (TGFα) are putative candidates for such a role, enhancing early follicular growth (Bendell and Dorrington, 1990) but inhibiting aromatization (Franks et al., 1988; Almahbobi et al., 1995). We have recently demonstrated that granulosa cells from both anovulatory and ovulatory patients with PCO express significantly higher levels of EGF receptors than granulosa cells from size-matched follicles of normal ovaries, as further evidence for the possible role of EGF/TGFα in the maintenance of PCOS (Almahbobi et al., 1998). However, the mechanism of action of EGF/TGFα on ovarian oestradiol biosynthesis in PCOS has not been defined. It is well known that the expression and activity of the aromatase enzyme are mainly gonadotrophin-dependent. Therefore, it is of interest to determine whether the inhibitory effect of EGF/TGFα is specifically related to interference in the action of gonadotrophins and their receptors, or directly related to enzyme expression and function. In a previous report, we have shown that granulosa cells from PCO contain significantly higher levels of follicle stimulating hormone (FSH) receptors than those from size-matched follicles of normal ovaries (Almahbobi et al., 1996). The aim of the present study was to investigate the mechanism which may mediate the action of EGF/TGFα in the regulation of ovarian activity.

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oestradiol concentrations were measured by means of radioimmunoassay (Acuson, Melbourne, Australia) and peripheral plasma levels were determined by competitive protein binding assay (Lucrin, 0.5–1 mg s.c.; Abbott Pharmaceuticals, Kurnell, Australia). Follicles were evaluated by clinical and endocrine manifestations of the syndrome (Trounson et al., 1981). In PCOS, the ovaries are enlarged with more than 10 follicles of ≥5 mm diameter in each. Other abnormalities of PCOS include anovulation, elevated androstenedione concentrations in serum (>6.5 nmol/l), LH:FSH ratios greater than 2 and reduced serum oestradiol concentrations (<0.2 nmol/l). These characteristics were variable in ovulatory patients with PCO and were frequently within the normal range for fertile women (Almahbobi et al., 1996).

Follicular aspirates were collected from anovulatory patients with PCOS just before laparoscopic electrocautery as part of treatment to induce ovulation for the purpose of conception in vivo. Samples were also collected from volunteers who were ovulatory with normal ovaries or PCO undergoing laparoscopic diagnosis for the investigation of their infertility. None of these patients received fertility drugs before follicular aspiration. Information on follicular number and size was recorded at the time of ultrasound examination 2 days before the sample collection. In anovulatory patients, follicular aspirates were collected when convenient and unrelated to any previous menses (Barnes et al., 1995; Szell et al., 1996). In the ovulatory groups of patients with normal ovaries or PCO, follicular aspiration was carried out on days 10–12 or 9–12 of the menstrual cycle respectively. The follicles were at immature stage with a size range of 2–9 mm (anovulatory patients with PCOS) and 2–10 mm (ovulatory patients with normal or PCO). However, not all follicles were necessarily aspirated.

In another group of patients, follicular aspirates were collected from mature pre-ovulatory follicles (Trounson et al., 1981) of patients undergoing ovulation induction with full stimulation with exogenous gonadotrophins for the recovery of mature oocytes for in-vitro fertilization (Calderon and Healy 1993). This group of patients is referred to as superovulated patients, regardless of their original aetiology. The treatments of these patients constituted pituitary down-regulation with gonadotrophin-releasing hormone (GnRH) agonist (Lucrin, 0.5–1 mg s.c.; Abbott Pharmaceuticals, Kurnell, Australia) and human menopausal gonadotrophin (HMG, Metrodin HP; Serono, French Forests, Australia) administration resulting in the growth and development of a cohort of ovulatory follicles. When at least three follicles of ~17 mm diameter were detected by vaginal guided ultrasound (Acuson, Melbourne, Australia) and peripheral plasma oestradiol concentrations were ≥3 nmol/l, 5000 IU of human chorionic gonadotrophin (HCG, Profasi; Serono) was given as a single i.m. injection to administer to complete follicular and oocyte maturation. Follicular aspiration (oocyte retrieval) was performed on day 2 or 3 and the remainder of the follicular fluid was poured into a 50 ml bottle for subsequent isolation of granulosa cells. In all cases, aspirates from several small follicles of each patient were pooled together, the distribution of collected samples from different patient groups, numbers and days of collection are shown in Table I. These studies were approved by the Monash Private Hospital Research and Ethics Committee.

Materials and methods

Table I. Collection of follicular aspirates from different patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Age (years)</th>
<th>Follicle number</th>
<th>Follicle size (mm)</th>
<th>Day of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4</td>
<td>27–38</td>
<td>11–23</td>
<td>2–10</td>
<td>10–12</td>
</tr>
<tr>
<td>Ov.PCO</td>
<td>15</td>
<td>24–38</td>
<td>34–60</td>
<td>2–10</td>
<td>9–12</td>
</tr>
<tr>
<td>Anov.PCO</td>
<td>7</td>
<td>28–36</td>
<td>40–65</td>
<td>2–9</td>
<td>N/A</td>
</tr>
<tr>
<td>Superovulated</td>
<td>40</td>
<td>25–41</td>
<td>9–34</td>
<td>12–28</td>
<td>11–15</td>
</tr>
</tbody>
</table>

Table I. Collection of follicular aspirates from different patients

Pooled aspirates were collected from ovulatory patients with normal ovaries (normal), polycystic ovaries (Ov.PCO) and anovulatory patients with polycystic ovaries (Anov.PCO) without prior treatment or after full stimulation with gonadotrophins (superovulated). The day of collection refers to the day of the cycle. N/A = not applicable.

Granulosa cell preparation and culture

All chemicals and reagents were purchased from Sigma Chemical Co (St Louis, MO, USA), unless otherwise specified. Granulosa cells were prepared from follicular aspirates as previously described (Almahbobi et al., 1996). In brief, follicular aspirates were centrifuged at 750 g for 5 min, the supernatant removed, the cell pellets pooled and then resuspended in 5 ml of Tissue Culture Medium 199 (TCM 199) supplemented with 25 mM HEPES, 50 mg/l penicillin, 50 mg/l streptomycin, 1.2 mg sodium bicarbonate and 0.1% bovine serum albumin (BSA). The granulosa cell suspension was dispersed by repeated pipetting for 30 s using a glass Pasteur pipette instead of an enzymatic method. This method allows only granulosa cells, but not (if any) theca or stroma cells, to be dispersed and also reduces the damage to receptors on the cell membrane. The cells were then purified by centrifugation onto a 100% Ficoll gradient (Histopaque 1077) and then washed and resuspended in medium. An aliquot of dispersed cells was used to assess cell number and viability by Trypan Blue exclusion.

Granulosa cells were plated in TCM 199 and maintained at 37°C in a humidified atmosphere of 5% CO2 in air for different periods appropriate to the purpose of each experiment as indicated. The medium was carefully removed by aspiration and fresh pre-warmed medium was added. For the study of aromatase activity in culture, cells were plated at a concentration of 1×104 viable cells per 25 μl in 48-multiwell culture dishes (Falcon, Becton Dickinson, USA). Prior to testing for aromatase activity, the cells were pre-incubated for 0 (freshly prepared), 24 (D1), 48 (D2) or 120 h (D5). The test period was for 3 h in the presence or absence of 10–7 M testosterone as substrate, with or without 20 ng/ml FSH, 20 ng/ml LH, 2 mM 8-Br-cAMP and 10 ng/ml EGF or 10 ng/ml TGFα. In some experiments, incubation time of freshly prepared cells was extended to 24 h in order to detect oestriadiol production of granulosa cells from small follicles of normal ovaries. Purified testosterone, human pituitary FSH and LH, 8-Br-cAMP, EGF and TGFα were diluted with sterile TCM 199 and added to appropriate wells in 10 μl volumes. The final volume of each well was 250 μl. Each test substance was repeated five times. At the end of the culture, the medium was collected for the measurements of oestriadiol and the cells were lysed with 50 μl 0.1 M sodium hydroxide and harvested for assay of protein concentration.

In other experiments designed for the quantification of LH using flow cytometry, granulosa cells were plated into 6-multiwell culture dishes (Falcon, Becton Dickinson, USA) at a concentration of ~4×105 cells/ml/well with media containing 10% fetal calf serum (FCS) and maintained for up to 5 days in the presence of testosterone (10–7 M), with or without EGF (10 ng/ml) or TGFα (10 ng/ml). The wells were carefully washed with pre-warmed media every 48h. At the end of the culture periods, the spent media were removed and...
the cells were washed with medium containing no FCS. Trypsin
tissue (1 ml) was added to each well and the plate was gently
subcultured until the cells reached confluence. The detached granulosa cells
were collected into tubes containing medium with FCS, washed
and transferred to Eppendorf tubes in 100 μl media for LHR
immunolabelling.

Oestradiol-17β and protein assays
The measurements of oestradiol in cell-free culture spent media were
based on a previously described radioimmunoassay (Carson et al.,
1986) using 10 000 c.p.m. per tube 2,4,6,7-3H-oestradiol (Amersham
International, Buckinghamshire, UK) and charcoal separation. Unla-
belled oestradiol [1,3,5(10 estratriene-3, 17β diol)] was used in serial
dilution range of 800 pg/100 μl to 3.13 pg/100 μl. Diluted spent
media (200 μl) was incubated with a rabbit anti-oestradiol antibody
(Ouchter oestradiol Y17) at a dilution of 1:15 000 overnight at 4°C.
After charcoal separation, the samples were counted with 2 ml scintillation liquid on a β-counter (Packard Instrument Co, Meriden,
USA). The sensitivity of the assay was 31.3 pg/ml. Proteins were
determined using the Bio-Rad DC colorimetric protein assay with a
BSA standard according to the instructions of the supplier (Bio-Rad
Laboratories Ltd, Mericles, USA).

Immunofluorescence labelling and microscopy
The immunolocalization and quantification of LHR was carried out
basically by a binding method with indirect detection through the
immunolabelling of the receptor-bound ligands. Granulosa cells that
were freshly prepared or following 5 days of culture were resuspended
in pre-cooled phosphate-buffered saline (PBS) containing 5% FCS
and left on ice for 30 min to allow the temperature of the cells to
decrease. The cells were then incubated with an excess amount of
human pituitary LH (200 ng/ml) for 45 min at 6°C with shaking
every 10 min (Haigler et al., 1978). The procedure allowed the ligand
to bind to the receptor but inhibited internalization of the receptor–
ligand complex. This was followed by extensive washes to remove
the unbound LH and the cells were processed, in suspension, for
indirect immunofluorescence labelling at 4°C (Almahbobi and Hall
1993; Almahbobi et al., 1998). Non-specific binding of secondary
antibody was reduced by incubation with 10% sheep serum (Oncogene
Research Products, Cambridge, USA) diluted in PBS/FCS for 30 min.
Monoclonal mouse anti-LH antibody was allowed to incubate for
30 min at a concentration of 10 μg/ml in PBS/FCS. Controls for non-
specific binding were determined using mouse negative control
immunoglobulin (IgG2a antibody (Dako, Glostrup, Denmark) instead
of the specific antibody. After incubation with the control or specific
antibody, granulosa cells within the Eppendorf tubes were thoroughly
washed with PBS/FCS. This was followed by incubation with 5 μg/ml sheep anti-mouse [F(ab)2 fragments] antibody conjugated to
fluorescein isothiocyanate (FITC) (Silenus Laboratories, Melbourne,
Australia) for 30 min. After extensive washes in PBS/FCS, the cells
were fixed in 1% paraformaldehyde in PBS for 10 min for immediate
analysis by flow cytometry, or mounted onto slides for microscopic
examination. In some experiments, cells that had been cultured on
coverslips (HD Scientific, NSW, Australia) over the 5 day period
were labelled in the same manner for microscopy without trypsiniz-
ation. Once the cells were fixed, a drop of Vectashield mounting
medium (Vector Laboratories, Inc, Burlingame, CA, USA) was placed
on slides, on which the coverslips were placed upside down. Examinations
were performed with a fluorescence microscope equipped for illumina-
tion using a mercury light source.

Flow cytometry
Labelled granulosa cells in 300 μl suspension were filtered through
75 μm mesh and analysed by flow cytometry basically as previously
described (Almahbobi et al., 1996, 1998). Cell suspensions were run
on a MO-FLO flow cytometer (Cytometry Inc, Fort Collins, CO,
USA). An argon ion laser (Coherent, Palo Alto, CA, USA) operating
at 488 nm was used to illuminate the cells. Forward angle light
scatter, right angle light scatter and FITC fluorescence (525 ± 10 nm
band pass filter) signals were collected for all samples. These were
all processed through a 3-decade logarithmic amplifier prior to digital
conversion. Fluorescence histograms of at least 5000 counts were
generated from a gate set on the granulosa cells in the forward angle
versus 90° light scatter histogram. Identification of granulosa cells
among other contaminating cells was by their characteristic large
size, high granularity, detectable autofluorescence (Almahbobi et al.,
1996) together with the negative labelling with CD45 (specific surface
marker of leukocytes), positive labelling with anti-EGF receptor
antibody (Almahbobi et al., 1998) and LH antibody labelling (the
present study). The mean fluorescence signals collected from a gated
cell population are expressed in numerical values. The values for
positive labelling were determined by subtraction of background
values obtained in control samples incubated with the control antibody
from the values obtained when the corresponding samples incubated
with the specific anti-LH antibody.

Statistical analysis
The data from steroid production experiments were calculated using
Microsoft Excel for Windows, version 5.0 for calculation of mean ±
SD from five replicate wells of each treatment within patient groups.
Comparisons between different groups of patients was undertaken
using Student’s paired t-test. Results of the LHR study, expressed as
the mean ± SD using Microsoft Excel Version 6.0, were analysed
by the Wilcoxon Signed Rank test for a non-parametric paired data
analysis. SPSS (Software Packages for the Statistical Sciences) for
Windows, version 6.0 (SPSS Inc, 1993) was used for both studies.
The statistical significance of individual experiments is shown in
the figure legends.

Results
Aromatase activity in PCO
Aromatase activity in granulosa cells as expressed by oestradiol
production was negligible without the addition of androgen
substrates in all cultures at any given time. The addition of
10^7 M testosterone to freshly prepared granulosa cells and
cells after 2 days in culture produced around 40- and 20-fold
increase (P < 0.001) in oestradiol production respectively
when compared with those without testosterone (data not
shown). These results confirm that human granulosa cells do
not synthesize androgens and that these granulosa cell cultures
were free of contaminating stroma and thecal cells. It should
be noted that there was no significant difference in basal and
stimulated oestradiol production between granulosa cells from
ovulatory and anovulatory patients with PCO (Figure 1A) and
therefore data were combined for subsequent analyses. Time-
dependent changes in basal aromatase activity showed a sharp
decline after 1 day of culture reaching about 15-fold less
oestradiol production compared with fresh cells. Oestradiol
production was maintained at this level during subsequent
days (Figures 1B). Interestingly, the basal oestradiol production
of freshly prepared and cultured granulosa cells from patients
with PCO was comparable with that of granulosa cells from
fully matured follicles of superovulated patients (Figure 1B).
Aromatase activity in PCOS

Figure 1. Aromatase activity in granulosa cells from polycystic ovaries (PCO). (A) Basal oestradiol production between freshly prepared granulosa cells from ovulatory (n = 7) and anovulatory (n = 5) patients with PCO after 3 h incubation. There were no significant differences and therefore the data were combined. (B) Time-dependent changes in basal aromatase activity in freshly prepared granulosa cells from PCO (solid bars, n = 10) after 3 h incubation pre-cultured from day 1 up to day 5, compared with granulosa cells from mature periovulatory follicles of superovulated patients (grey bars, n = 10). (C) Aromatase activity of granulosa cells from PCO and size-matched follicles of normal ovaries (open bars, n = 3) incubated for 24 h. Each bar represents the mean ± SD of number of patients (n) in five replicate wells. *P < 0.001.

In addition, this ability of granulosa cells from patients with PCO to produce oestradiol was significantly (P < 0.001) greater than that of granulosa cells obtained from normal ovaries, incubated under both basal and stimulated conditions (Figure 1C).

Aromatase activity in response to gonadotrophins in culture

To determine whether granulosa cells from patients with PCO are responsive to gonadotrophins, freshly prepared granulosa cells were incubated with FSH or LH for 3 h in the presence of testosterone as a substrate. In these short-term incubations, the addition of gonadotrophins failed to stimulate oestradiol production by the granulosa cells from patients with PCO and superovulated patients (Figure 2). In contrast, when granulosa cells were cultured for at least 2 days, the addition of FSH or LH to the cultures for 3 h induced a significant (P < 0.001) increase in oestradiol production. The stimulatory effect of gonadotrophins on aromatase activity was enhanced after 5 days in culture (Figure 2). Once again, the gonadotrophin-stimulated oestradiol production of granulosa cells from patients with PCO was identical to those of granulosa cells from superovulated patients.

Effects of EGF/TGFα on aromatase activity

In freshly prepared granulosa cells from patients with PCO and superovulated patients, there was no inhibitory effect of EGF or TGFα on basal aromatase activity (Figure 3A). Since there was no stimulation in the presence of gonadotrophins, the basal oestradiol production was unaffected by EGF/TGFα. Because there was no difference between the effect of EGF and TGFα on aromatase activity, the data were combined for subsequent analyses. In granulosa cells cultured for at least 2 days, EGF/TGFα significantly (P < 0.001) inhibited gonadotrophin-stimulated oestradiol production but not basal aromatase activity and this was more pronounced in cultures after 5 days before adding EGF/TGFα for the 3 h test (Figure 3B).

Mechanism of action of EGF/TGFα on aromatase activity

Multiple lesions

In order to determine the mechanism which may regulate the effect of EGF/TGFα on gonadotrophin-induced aromatase activity, freshly prepared and cultured granulosa cells were incubated with or without EGF/TGFα in the presence or absence of exogenous 8-Br-cAMP. In contrast to the lack of aromatase stimulation by gonadotrophins in freshly prepared cells (Figure 3A), there was a significant (P < 0.001) increase in oestradiol production in response to 8-Br-cAMP of the cells obtained from the patient groups, with polycystic ovaries and who were superovulated (Figure 4). After 2 days in culture, the increased oestradiol production with 8-Br-cAMP was more pronounced than that with FSH or LH shown in Figure 2.
Most interestingly, the increase in oestradiol production in response to 8-Br-cAMP was also significantly \( (P < 0.001) \) inhibited by EGF/TGF\( \alpha \) when added to either fresh or cultured cells (Figure 4).

**EGF/TGF\( \alpha \) inhibits LHR binding in vitro**

The quantification of LHR in granulosa cells of different groups of patients was carried out using flow cytometric analysis. The identification and gating of the granulosa cell fraction within the suspended cell preparations were based on a previously described procedure (Almahbobi et al., 1996, 1998). Since granulosa cell preparations were pre-cultured for several days before flow cytometry, most of the contaminating blood cells were removed, due to several washes, from these preparations as revealed by the forward light scatter analysis (Figure 5A). The labelling of LHR within the gated population of granulosa cells from both PCO and normal ovaries showed a heterogenous pattern of labelling. Figures 5B and 5C show representative histograms of fluorescence signals collected from granulosa cells of PCO. The histograms show the relative intensity of labelling and the number of labelled cells within the gated population of granulosa cells shown in Figure 5A. The levels of displacement of granulosa cell population indicate the intensity of the fluorescence signals collected per individual cell. Figure 5B shows non-specific binding using control antibody. When the specific antibody was used the whole cell population, which appeared on the left-hand side of the vertical cut-off line, was partially displaced to the right side on the fluorescence scale (Figure 5C). A fraction of the positively labelled cells showed strong labelling intensity with a maximum shift to the right hand side of the cut-off line (Figure 5C).

The mean \( \pm \) SD of LHR-positive labelling in granulosa cells cultured for 5 days with or without EGF/TGF\( \alpha \) is shown in Figure 6. Less consistent and significant changes in LHR were observed in 1 and 2 day cultures (data not shown). The positive LHR labelling was obtained by subtracting the control values from the values of corresponding samples incubated with the specific antibody. Interestingly, the LHR binding in granulosa cells from patients with PCO was significantly \( (P < 0.001) \) less than that seen in granulosa cells of size-matched follicles from normal ovaries or pre-ovulatory follicles of superovulated patients. In addition, both EGF and TGF\( \alpha \) significantly inhibited the binding of LHR in granulosa cells from all patient groups (superovulated and normal patients, \( P < 0.05 \); patients with polycystic ovaries, \( P < 0.001 \)) (Figure 6). More interestingly, granulosa cells from PCO were more sensitive to the inhibitory effect of EGF and TGF\( \alpha \) on LHR (50%) compared with granulosa cells of matched-size follicles from normal ovaries (16%) and to a lesser extent to granulosa cells from superovulated patients (40%). Examination of the freshly prepared (Figure 6 inset) and cultured granulosa cells under the fluorescence microscope revealed that LHR-positive staining was confined to the cell membrane.
Aromatase activity in PCOS

Figure 5. Flow cytometric analysis of luteinizing hormone receptor (LHR) in granulosa cells. (A) Forward light scatter analysis revealed a distinct population of granulosa cells. (B) and (C) Representative histograms of fluorescence signals collected per individual cell of at least 5000 granulosa cells from PCO. (C) The strong labelling of LHR is clearly shown in a cell fraction displacing to the right-hand side of the cut-off indicated by the vertical line. (B) Non-specific binding using control antibody was very low in the corresponding control samples. 90 LS = right angle light scatter; FALS = forward angle light scatter.

This was supported by the staining of cell clumps characteristic of undispersed granulosa cells (data not shown). The staining intensity of individual cells within the granulosa cell population and amongst individual patients within a specific patient group was varied. All control samples revealed negative staining.

Discussion

PCOS is a heterogeneous disease with many contradictory features particularly the inhibition of ovarian cyclic oestrogen production. Although an intrinsic defect in aromatase expression or activity is unlikely to be the case in PCOS, the clear ambiguity in the function of this enzyme, in different in-vivo and in-vitro conditions, makes it difficult to determine the normality of aromatization in PCOS. It has been reported that within the range of PCO follicles (<10 mm), aromatase enzyme is not normally expressed (Erickson et al., 1979; Jakimiuk et al., 1998) and that the in-vitro production of oestradiol by PCO granulosa cells is due to cell differentiation in long-term (48 h) cultures (Erickson et al., 1979). However, the aromatase enzyme was immunolocalized in human granulosa cells within the same range of follicle size (Sasano et al., 1989) and also those of <1 mm diameter (Inkster et al., 1991). It is also possible that differentiation of granulosa cells, or even development of new growing follicles may occur in vivo after the administration of gonadotrophins, explaining the response of PCO granulosa cells (Almahbobi and Trounson 1996). Nevertheless, differentiation of granulosa cells should not lead to excessive oestradiol production by PCO granulosa cells when compared with granulosa cells from normal ovaries as previously reported (Haney et al., 1986; Almahbobi et al., 1996; Pierro et al., 1997; for more references see Almahbobi

Figure 6. Effects of EGF/TGFα on LH receptor binding in cultured granulosa cells from different patients. The mean ± SD of LH receptor binding in granulosa cells from patients with PCO (solid bars) after 5 days in culture was significantly (**P <0.001) less than that in granulosa cells from normal (open bars) or superovulated (grey bars) patients. The presence of EGF or TGFα in culture significantly reduced the labelling of LH receptors in granulosa cells from patients of all different groups. Granulosa cells from PCO were significantly (**P <0.001) more sensitive to the inhibitory effect (50%) of EGF/TGFα on LH receptor binding compared to granulosa cells of matched-size follicles from normal ovaries (*P <0.05, 16%) and superovulated patients (*P <0.05, 40%). Each bar represents the mean ± SD of 7 superovulated patients, 3 patients with normal ovaries, and 5 patients with PCO. Inset: Fluorescence microscope revealed the LH receptor positive staining which was confined to the cell membrane of granulosa cells. Original magnification ×400.
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In order to clarify this confusion, we have investigated aromatase activity in freshly prepared cells incubated for short period (3 h) under basal conditions (no added serum or gonadotrophins). It was interesting to note that granulosa cells from immature follicles (2–10 mm) of PCO contain high aromatase activity, comparable with that seen in fully mature pre-ovulatory follicles of superovulated patients and as much as 13 times more activity than those of normal ovary. A recent report showing that PCO granulosa luteal cells, after treatment with gonadotrophins for the induction of superovulation, also had higher levels of aromatase activity in vitro when compared with non-PCO cells (Pierro et al., 1997).

Since the concentrations of bioactive FSH are normal in PCO (Erickson et al., 1992) and PCO granulosa cells contain significantly higher concentrations of FSH receptors than those of normal ovaries (Almahbobi et al., 1996), it is possible that granulosa cells of PCO express high values of aromatase enzyme. However, in vivo aromatization is potentially inhibited, possibly by the intraovarian growth factors EGF/TGFα.

In vitro, the inhibitory effect of EGF/TGFα was removed. The values of oestradiol production were high and comparable with those of pre-ovulatory follicles from superovulated patients. Moreover, both types of cells did not respond to gonadotrophins during the first 24 h in culture. This may indicate that granulosa cells from PCO follicles may be functioning at near maximal aromatization. With subsequent time in culture, the cells lost the influence of the inherited in vivo effects, aromatization sharply dropped to normal values and the cells recovered their normal responsiveness to gonadotrophins. In contrast to EGF, it has been shown that insulin-like growth factor I enhances the FSH-induced expression of aromatase in cultured human granulosa cells from superovulated patients (Steinkampf et al., 1988). Nevertheless, PCOS is a heterogenous syndrome whereby multiple factors may act separately or synergistically.

Three important points emerge from this study regarding the regulation of aromatase activity in human granulosa cells. First, in freshly prepared cells, only 8-Br-cAMP but not FSH or LH could stimulate aromatase activity. This indicates that the failure of gonadotrophins to induce aromatase stimulation in fresh cells is due to a lesion at a step preceding cAMP production. Secondly, regardless of the culture period or the cell types, EGF/TGFα suppressed only the stimulated aromatase activity but not the basal levels of aromatization (Steinkampf et al., 1988). This may indicate that the high levels of oestradiol production in both types of cells were in fact basal activity of highly expressed enzyme. Thirdly, the fact that EGF/TGFα inhibited aromatase stimulation in the presence of an excess amount (or at least stimulatory amount) of 8-Br-cAMP indicates that the site of the inhibitory action of EGF/TGFα is at a post-cAMP production step. In support of this, it has been shown that in cultured rat granulosa cells, EGF not only reduces the FSH-stimulated adenylate cyclase activity but also increased catabolism of cAMP (Knecht and Catt, 1983), therefore exerting multiple sites of action. We suggest that while the levels of basal aromatization appears totally related to the amount of the expressed enzyme and are not affected by the intraovarian inhibitors such as EGF/TGFα, the hormonally stimulated aromatization requires cAMP and is regulated by EGF/TGFα.

The presence of LHR in human granulosa cells of follicles <10 mm is not surprising particularly during day 9–12 of the menstrual cycle (McNatty et al., 1992). LHR analysis showed that not all granulosa cells were positively labelled and the labelling intensity was variable. The intensely labelled cells were probably derived from the larger follicles within individual pooled samples. The inhibition of LHR binding, detected by immunolabelling, in human granulosa cells by EGF/TGFα confirms previous studies using cultured rat granulosa cells (Mondshein and Schomberg, 1981; Knecht and Catt, 1983) and highlights the possible role of these growth factors in the maintenance of PCOS. This view was supported by the low levels of LHR labelling in PCO granulosa cells comparing to both stimulated and unstimulated cells from normal ovaries. This was confirmed by our previous study showing that the capacity of PCO granulosa cells to produce progesterone in culture was significantly less than those from normal ovaries (Almahbobi et al., 1996). Our data show that there is no significant difference between LHR detected in granulosa cells from small follicles of normal ovaries and those from mature follicles of superovulated patients. This is probably due to the rapid decline of LHR mRNA of pre-ovulatory follicles that normally occurs after the LH surge in vivo (Segaloff et al., 1990) in superovulated patients. An interesting and novel observation of the present study was that PCO granulosa cells were more sensitive to the inhibitory effects of EGF/TGFα on LHR. It appears that the high levels of EGF receptors in granulosa cells of PCO (Almahbobi et al., 1998) may explain the hyper-sensitivity of these cells to the action of EGF/TGFα. Because a similar hypersensitivity has not been found in terms of the inhibition of oestradiol production (Mason et al., 1990; the present results), it appears that the amplification of EGF/TGFα action occurs in a selective manner.

The above results, together with others previously reported, suggest that in PCOS, FSH concentrations and their related ovarian functions are normal, including the selection of pre-ovulatory follicles (Erickson, 1996). However, the late follicular maturation of a selected follicle to achieve dominance is blocked. The latter process requires large amounts of oestrogen for multiple purposes such as follicular growth and the formation of LHR in granulosa cells (Richards et al., 1976; Segaloff et al., 1990). In fact, the blockade of follicular growth in PCO occurs around the time when granulosa cells acquire LHR and hence become responsive to this hormone (McNatty et al., 1992). LH is a principle regulator of aromatization (Hsueh et al., 1984) in the late follicular stage and its main role is to enhance late maturation of the selected follicle to ensure dominance and ovulation (Richards, 1994). In conclusion, most of the PCOS characteristics indicate hyperactivity of ovarian function (Almahbobi and Trounson, 1996) but the inhibition of cyclic ovarian oestradiol production and LHR formation by EGF/TGFα is a mechanism likely to be operative in PCOS, leading to the blockade of follicular dominance and anovulation.
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