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Aromatase mRNA expression in individual follicles from polycystic ovaries

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Polycystic ovary syndrome (PCOS) is a common reproductive disorder characterized by arrested follicular development prior to selection of a dominant follicle. Dominant follicles produce large amounts of oestradiol but PCOS follicles do not. With several potential aromatase ($P450_{AROM}$) inhibitors in follicular fluid, the question arises whether $P450_{AROM}$ is expressed in PCOS granulosa cells, but the activity is inhibited, or whether $P450_{AROM}$ is not expressed in PCOS. The purpose of the present study was to determine whether $P450_{AROM}$ mRNA expression is altered in PCOS and to correlate $P450_{AROM}$ mRNA expression in individual follicles with aromatase stimulatory bioactivity and oestradiol in the follicular microenvironments. $P450_{AROM}$ mRNA was measured in individual follicles from 16 PCOS and 48 regularly cycling control women by quantitative polymerase chain reaction (PCR) and correlated with follicular fluid oestradiol concentrations and aromatase stimulating bioactivity measured by the rat granulosa cells aromatase bioassay. Follicular fluid oestradiol was low in all control follicles <7 mm in diameter. Some follicles ⩾7 mm contained elevated oestradiol values ($P < 0.01$) and all had an androstenedione:oestradiol ratio of <4. Only in granulosa cells from follicles ⩾7 mm with an androstenedione:oestradiol ratio of <4 were $P450_{AROM}$ mRNA levels increased ($P < 0.05$). These same follicles also contained increased levels of aromatase stimulating bioactivity whereas follicles <7 mm or with androstenedione:oestradiol ratio of >4 contained little or no bioactivity. All PCOS follicles contained low levels of oestradiol , $P450_{AROM}$ mRNA and aromatase stimulating bioactivity similar to size-matched control follicles. These data indicate that $P450_{AROM}$ mRNA expression and oestradiol production begin in developing follicles when they reach ~7 mm in diameter. Oestradiol production is low in PCOS follicles because there is insufficient aromatase stimulating bioactivity to increase $P450_{AROM}$ mRNA expression.

Key words: aromatase/mRNA/oestradiol/PCOS

Introduction

Polycystic ovary syndrome (PCOS) is a common and heterogeneous reproductive disorder in women of childbearing age (Polson *et al.*, 1988) characterized by hyperandrogenism and chronic anovulation (Goldzieher and Axelrod, 1963; Yen, 1980). Despite considerable controversy surrounding diagnostic criteria for PCOS (Lobo, 1995), a common feature in polycystic ovaries is arrested follicular development at the stage when selection of the dominant follicle should normally occur (Erickson and Yen, 1984; Gougeon, 1986). Consequently a large number of small antral follicles (4–7 mm diameter) accumulate in the ovaries of PCOS women and a dominant follicle destined for ovulation rarely develops (Goldzieher, 1981; Erickson and Yen, 1984). The mechanism underlying the arrested follicular growth in PCOS is unknown.

An essential feature of the dominant follicle is an oestrogenic microenvironment characterized by an androstenedione:oestradiol ratio of <4 (McNatty *et al.*, 1975). In polycystic ovaries the follicular fluid oestradiol concentration is low compared with that in dominant follicles despite high levels of androstenedione (San Roman and Magoffin, 1992; Erickson *et al.*, 1992). Thus, there is no lack of substrate for aromatization. When granulosa cells are removed from the in-vivo follicular milieu of the polycystic ovary and treated with follicle stimulating hormone (FSH) in serum-free cell culture, they secrete normal or increased amounts of oestradiol compared to granulosa cells from control ovaries (Erickson *et al.*, 1992; Mason *et al.*, 1994). Similarly, follicle development and oestradiol production can be stimulated in women with PCOS using exogenous FSH treatment (Fauser, 1994). These observations demonstrate that the granulosa cells in polycystic ovaries contain functional FSH receptors and are capable of responding to the FSH signal with appropriate levels of oestradiol production.

Taken together, the data suggest that PCOS follicles contain an endogenous inhibitor of oestradiol production. There are several proteins in follicular fluid with the potential to inhibit oestradiol production such as a high molecular weight FSH receptor binding inhibitor (Lee *et al.*, 1993), inhibin-α subunit precursor (Schneyer *et al.*, 1991), insulin-like growth factor binding proteins (IGFBPs) (Ui *et al.*, 1989) epidermal growth

factor (EGF) (Mason *et al.*, 1990) and tumour necrosis factor-α (TNF-α) (Rice *et al.*, 1996). No data are available demonstrating that the physiological concentrations of these peptides that are present in follicular fluid can significantly inhibit oestradiol production in PCOS. Analysis of FSH receptor activation inhibitors in follicular fluid from women with PCOS demonstrates that inhibition of FSH action at the level of the FSH receptor does not play an important role in PCOS (Schipper *et al.*, 1997). Recently, we have shown that 5α-androstane-3,17-dione, a competitive inhibitor of $P450_{AROM}$ activity, is present in abnormally high concentrations in PCOS follicular fluid that can maximally inhibit oestradiol production by human granulosa cells *in vitro* (Agarwal *et al.*, 1996). Thus, the potential exists that if $P450_{AROM}$ is expressed in PCOS granulosa cells, its activity is suppressed.

There are no published data on $P450_{AROM}$ mRNA expression during follicular development in PCOS. Thus, it is unclear whether a low level of $P450_{AROM}$ mRNA is expressed in PCOS granulosa cells, but the activity is inhibited, or if $P450_{AROM}$ mRNA is not expressed in PCOS. The purpose of the present study was to determine whether $P450_{AROM}$ mRNA expression is altered in PCOS and to correlate the expression of $P450_{AROM}$ mRNA in individual follicles with the amounts of aromatase stimulatory bioactivity and oestradiol in the follicular microenvironments.

Materials and methods

Subjects

Ovarian tissue specimens were obtained from 16 follicular phase women with PCOS undergoing electrocauterization of the ovarian surface or wedge resection for treatment of their infertility. Control tissues were obtained from 48 regularly cycling pre-menopausal women undergoing total abdominal hysterectomy and bilateral oophorectomy for non-ovarian indications unrelated to the study. Women with PCOS were identified based on a history of oligo/amenorrhoea, hirsutism, and typical morphological appearance of polycystic ovaries (normal or enlarged ovarian volume with multiple subcapsular cysts $<$8 mm in diameter) at laparotomy or laparoscopy with no evidence of hyperprolactinaemia, Cushing's syndrome, congenital or non-classical adrenal hyperplasia, thyroid disease, or hormone secreting tumours. All subjects had not received hormonal therapy or ovarian suppression for at least 3 months prior to obtaining the samples. Informed consent was obtained from all subjects participating in the study as approved by the Ethics Committee at the University School of Medicine in Lublin. These studies were also approved by the IRB at Cedars-Sinai Medical Center.

Follicular fluid and granulosa cell collection

The ovarian specimens were immediately placed into ice-cold Medium-199 (Gibco BRL, Gaithersburg, MD, USA) containing 25 mM HEPES and 1 mg/ml bovine serum albumin (BSA). After washing off the blood, the ovaries were placed under a dissecting microscope and the follicular fluid was completely aspirated from the visible follicles using a Hamilton syringe. The follicular fluid volume was measured and the granulosa cells were collected by centrifugation for 5 min at 250 *g*. The follicular fluid was frozen at –80°C until hormone assays were performed. The follicle diameter was calculated from the volume of aspirated fluid. The follicle was opened with microscissors and the granulosa cells were gently scraped from the follicle wall with a platinum loop and collected by flushing with medium. The granulosa cells were centrifuged and the pellet was pooled with the granulosa cells collected from the follicular fluid. The isolated granulosa cells were frozen at –80°C until nucleic acids and protein were extracted.

Steroid radioimmunoassay

The concentrations of androstenedione and oestradiol in the follicular fluids were measured by specific radioimmunoassays as previously described (Magoffin and Erickson, 1982).

DNA assay

Total cellular DNA and total RNA were isolated from the granulosa cells of individual follicles using Tri Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. The DNA pellet was dissolved in 50 µl of PBS buffer (0.1 M $NaPO_4$, 0.15 M NaCl, pH 7.4) at 37°C for 10 min. The DNA concentration of the samples was measured by a sensitive fluorescence assay as previously described (Downs and Wilfinger, 1983). Briefly, 50 µl of sample was added to 1.5 ml of 100 ng/ml Hoechst 33258 dye (Sigma, St Louis, MO, USA) and the fluorescence was then measured in a fluorometer (Hoefer Scientific, San Francisco, CA, USA). Sample concentrations were interpolated from a standard curve calculated by linear regression of the fluorescence of known concentrations of Herring sperm DNA.

$P450_{AROM}$ mRNA assay

Cytochrome P450 aromatase ($P450_{AROM}$) mRNA was measured by a quantitative assay based on reverse transcribing the mRNA into cDNA with reverse transcriptase (RT) then amplification of the cDNA using the polymerase chain reaction (PCR). The total RNA isolated with Tri Reagent was resuspended in 20 µl DEPC-treated water, then frozen at –80°C. Aliquots of RNA (4 µl) were transcribed into cDNA by incubating (37°C) for 30 min in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dATP, 1mM dCTP, 1 mM dGTP, 1 mM dTTP, 5 µg oligo$(dT)_{12–18}$ (Pharmacia, Piscataway, NJ, USA), 20 U RNAsin (Promega, Madison, WI, USA) and 200 IU Maloney Murine Leukaemia virus–reverse transcriptase (M-MLV RT; Gibco BRL) in a total volume of 20 µl. The reaction was then heated to 95°C (5 min) and cooled to 4°C. 1 pg of mutant control DNA, 50 pmol of each PCR primer, 8 µl of 10× PCR buffer (100 mM Tris–HCl (pH 8.3), 500 mM KCl), 9.6 µl of 25 mM $MgCl_2$, 10 µCi [^{32}P]-dCTP (3000 Ci/mmol; Dupont NEN, Boston, MA, USA), and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were added and the volume adjusted to 100 µl. The cDNA was amplified for 25 cycles (94°C for 30 s; 55°C for 20 s; 72°C for 30 s) in a thermal cycler. The amplification products were ethanol precipitated and digested with *Pvu*I to cut the control products, then separated on a 2% agarose gel. The DNA was visualized with ethidium bromide staining and the bands were cut from the gel and counted in a scintillation counter. The c.p.m. in the bands amplified from the cellular mRNA were normalized to the c.p.m. in the bands amplified from the mutant DNA to control for procedural variations. The data were also normalized to total cellular DNA to control for variations in the number of granulosa cells in each sample.

The oligonucleotide primers were synthesized in our laboratory using an Applied Biosystems model 391 DNA synthesizer (Foster City, CA, USA). $P450_{AROM}$ cDNA was amplified using primers corresponding to bases 391–410 and 911–930 of the published sequence (Corbin *et al.*, 1988) that amplified a specific 540 bp fragment. To control for PCR variations, a G was substituted for a T at base 663 by site-directed mutagenesis (Horton *et al.*, 1990) to introduce a unique *Pvu*I site. The control template (1 pg) was included

Table I. Follicular fluid oestradiol concentrations. Values are given as mean ± SEM, unless otherwise indicated

Follicle diameter (mm)	Control oestradiol (ng/ml)	*n*	Polycystic ovary syndrome (PCOS) oestradiol (ng/ml)	*n*
3	6.5 ± 1.4	6	11.6 ± 4.7	14
4	11.1 ± 2.1	11	9.0 ± 2.1	12
5	10.4 ± 2.5	16	7.1 ± 1.4	10
6	51.5 ± 22.0	15	10.7 ± 3.0	9
7	101.6 ± 44.6	16	102 (7.7–192)[a]	2
8	397.0 ± 232.1*	8	–	0

[a]Mean with range in brackets.
*P <0.01 compared with 3, 4, or 5 mm.

in each PCR reaction and all samples for each experiment were amplified at the same time.

Aromatase-stimulating bioactivity assay

The aggregate ability of substances contained in the follicular fluids to stimulate oestradiol production was measured using the rat granulosa cell aromatase bioassay as previously described (Jia *et al.*, 1986). Briefly, follicular fluid samples were diluted with an equal volume of 12% polyethylene glycol (PEG), incubated at 4°C for 30 min, then centrifuged at 2000 *g* for 5 min. The resulting supernatant was used in the bioassay. A standard curve was prepared using recombinant human FSH obtained from the National Hormone and Pituitary Program. Aliquots of PEG-treated follicular fluid and standards were incubated with granulosa cells obtained from immature oestrogen-treated Sprague–Dawley rats as previously described (Jia *et al.*, 1986). Oestradiol in the medium was measured by radioimmunoassay. The data were corrected for the oestradiol content in the follicular fluid samples tested by subtracting the amount of endogenous oestradiol in the sample from the total oestradiol measured prior to calculating the bioactivity present in the sample. In the majority of samples the endogenous oestradiol was negligible. PEG-treated serum from women using oral contraceptive pills was used as a negative control. The limit of detection was 0.2 mIU FSH equivalents/ml.

Statistical analysis

Differences between groups were analysed by analysis of variance followed by Tukey's test. $P < 0.05$ was considered to be significant.

Results

Oestradiol and androstenedione concentrations in the follicular fluid

The oestradiol and androstenedione concentrations were measured in all of the follicular fluid samples to characterize the steroidogenic milieu of each of the follicles. As shown in Table I, mean oestradiol concentrations were very low in the follicular fluid of control follicles 7 mm in diameter and smaller. Beginning with 8 mm follicles, the mean oestradiol values increased significantly, indicating that the granulosa cells in at least some of the follicles contained $P450_{AROM}$ activity. The estradiol concentrations in the follicular fluid of PCOS follicles were very low in all but one 7 mm follicle. There were no significant differences in mean estradiol levels between PCOS follicles and the size matched control follicles.

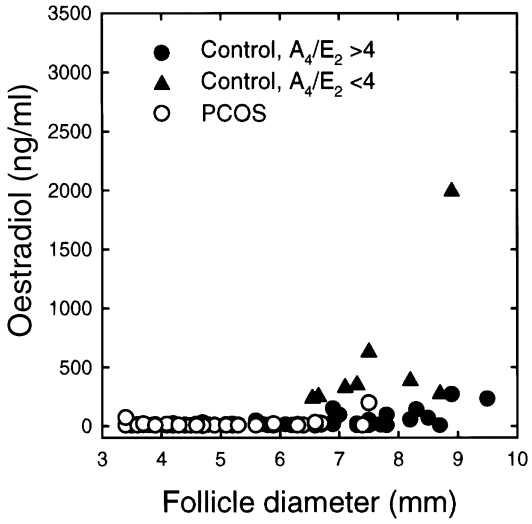

Figure 1. Follicular fluid oestradiol concentrations in 3–10 mm follicles from control and women with polycystic ovary syndrome (PCOS). Oestradiol was measured in the follicular fluids of 47 individual follicles from 19 women with PCOS and 76 individual follicles from 39 regularly cycling control women by specific radioimmunoassay.

When the follicular fluid oestradiol concentrations were plotted as a function of follicle diameter (Figure 1), it became apparent that the oestradiol concentrations were low in the majority of follicles of all sizes. Only a few of the follicles >6.5 mm had levels of oestradiol >200 ng/ml. Beginning at 6.6mm the follicles with an androstenedione:oestradiol ratio of <4 showed a progressive rise in oestradiol concentrations as follicle diameter increased, indicating that $P450_{AROM}$ activity first appears in follicles that are >6 mm. Interestingly, there were two follicles (8.2 and 8.7 mm) with androstenedione:oestradiol ratios of <4 that appeared not to follow the pattern of increasing oestradiol with larger follicle diameters. In both cases these were cohort follicles, having been obtained from ovaries with a larger dominant follicle and suggesting that these follicles were growth retarded relative to the dominant follicles.

In order for follicles to secrete significant amounts of oestradiol they must produce adequate levels of androstenedione. To determine if adequate amounts of androstenedione substrate were present in the follicles studied, the androstenedione concentrations in the follicular fluid were measured (Figure 2). As shown in Figure 2A, there was no difference between the mean androstenedione concentration in PCOS follicles compared to control follicles with androstenedione: oestradiol ratios of >4. In follicles with androstenedione: oestradiol ratios of <4 the mean androstenedione concentration was significantly decreased by ~30%. There was considerable variability in the androstenedione concentrations between follicles of similar diameter. In control follicles there was a trend toward increased androstenedione levels in the follicular fluid of larger follicles ($P = 0.012$); however, the correlation was weak ($r = 0.415$). In PCOS follicles there was a large variation in androstenedione concentrations, especially in small follicles. There was no correlation between follicular fluid androstenedione concentrations and follicle diameter ($P = 0.327$).

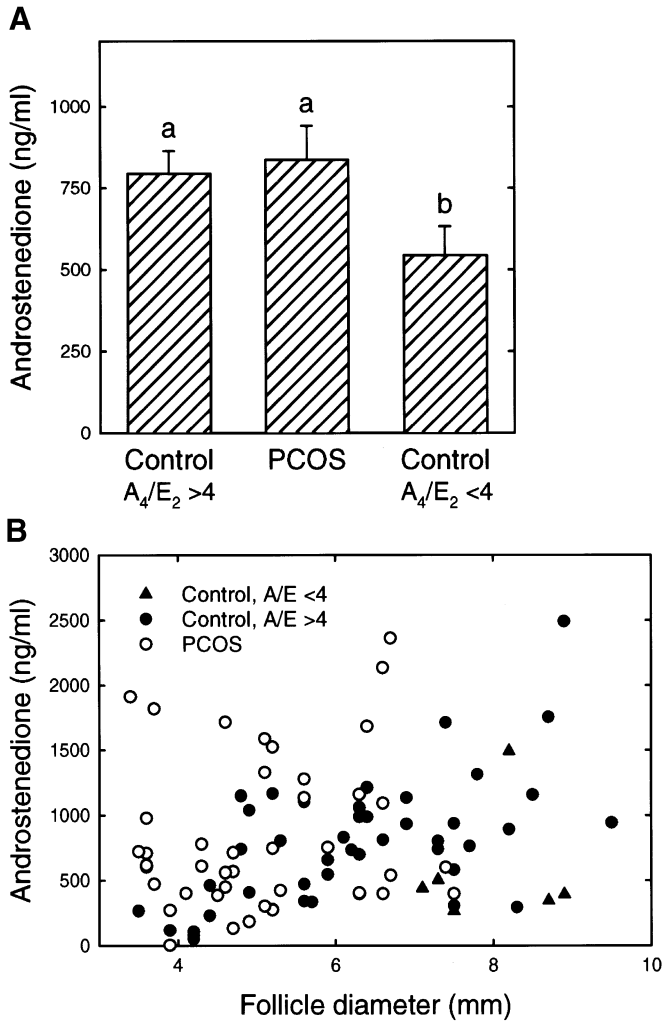

Figure 2. Androstenedione concentrations in the follicular fluid from control and women with polycystic ovary syndrome (PCOS). Androstenedione was measured in the follicular fluids of 41 individual follicles from 16 women with PCOS and 72 individual follicles from regularly cycling control women, 62 women with androstenedione:oestradiol ratios of >4 in the follicular fluid and nine women with androstenedione:oestradiol ratios of <4. (**A**) The data are given as the mean ± SEM. Bars with different letters are significantly different (P <0.05). (**B**) The data for individual follicles are plotted as a function of follicle diameter.

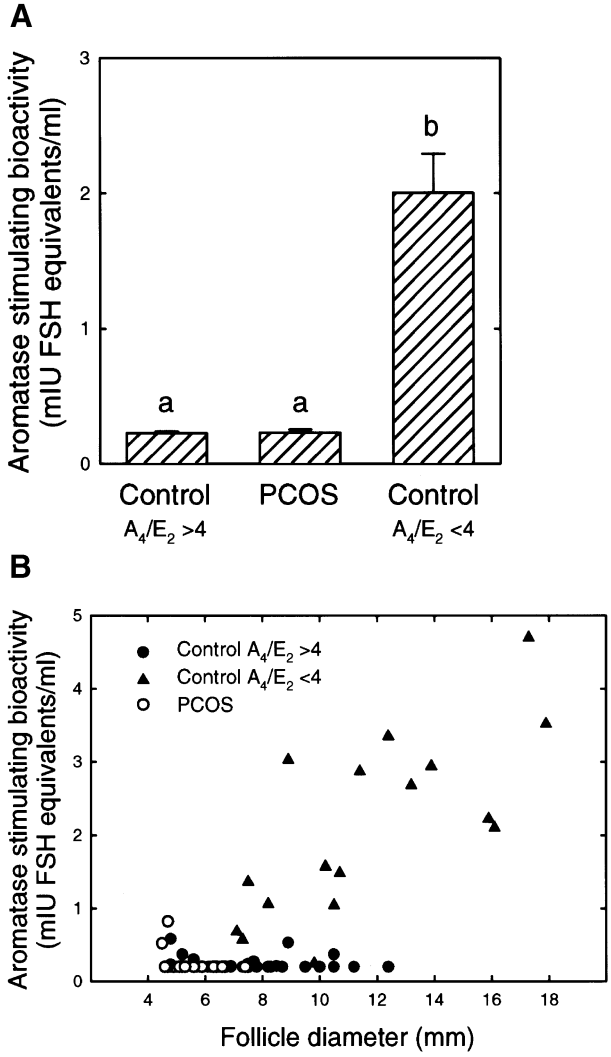

Figure 3. Aromatase stimulating bioactivity in the follicular fluid of regularly cycling women and women with polycystic ovary syndrome (PCOS). Aromatase stimulating bioactivity was measured in the follicular fluid of 29 follicles ⩾4.5 mm from 12 women with PCOS and 60 follicles >4.5 mm from 41 regularly cycling control women., 19 follicles had androstenedione:oestradiol ratios of <4 in the follicular fluid and 41 follicles had androstenedione:oestradiol ratios of >4. (**A**) The data are given as the mean ± SEM. Bars with different letters are significantly different (P <0.001). (**B**) The data for individual follicles are plotted as a function of follicle diameter.

Aromatase stimulating bioactivity in follicular fluid

The ovarian microenvironment can contain FSH together with a variety of growth and differentiation factors that are thought to be critical for stimulation of aromatase in the granulosa cells of dominant follicles (Zeleznik and Fairchild-Benyo, 1994). We next investigated the possibility that the aggregate amount of aromatase stimulating bioactivity in PCOS follicles might be abnormally low and thus fail to increase oestradiol production. As shown in Figure 3A, the mean aromatase stimulating bioactivity in the follicular fluid of control follicles with androstenedione:oestradiol ratios of >4 was markedly lower that in the control follicles with androstenedione:oestradiol ratios of <4. The mean aromatase stimulating bioactivity in the follicular fluid of PCOS follicles was equivalent to control follicles with androstenedione:oestradiol ratios of >4. The aromatase stimulating bioactivity for individual follicles is shown in Figure 3B. In control follicles with androstenedione:oestradiol ratios of >4 the aromatase stimulating bioactivity values were low. In control follicles beginning at ~7 mm in diameter there were increased levels of aromatase stimulating bioactivity in the follicular fluid of follicles with androstenedione:oestradiol ratios of <4. High levels of aromatase stimulating bioactivity were consistently measured in dominant follicles. In PCOS follicles the aromatase stimulating bioactivity was low, similar to control follicles with androstenedione:oestradiol ratios of >4.

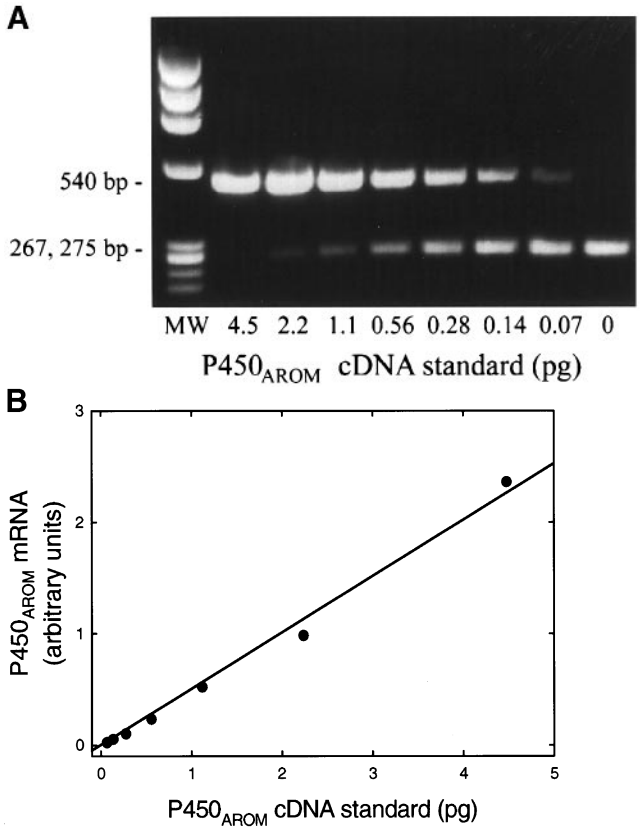

Figure 4. Reverse transcription–polymerase chain reaction (RT–PCR) assay for human $P450_{AROM}$ mRNA. Increasing concentrations of linear full-length human aromatase cDNA (Corbin *et al.*, 1988) were amplified by PCR using specific primers and incorporating [α-^{32}P]-dCTP. A specific control cDNA that was modified by site-directed mutagenesis to introduce a unique *Pvu*I site was included in the amplification reaction. The amplification products were digested with *Pvu*I and separated on a 2% agarose gel (**A**). The native (540 bp) and control (267 and 275) bands were excised from the gel and counted in a β-counter. (**B**) represents the radioactivity present in the native band as a function of the amount of $P450_{AROM}$ cDNA initially added to the reaction after normalization to the control bands to control for variations in PCR amplification.

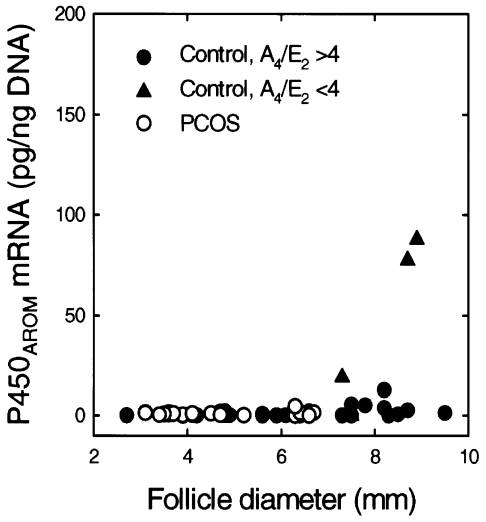

Figure 5. Cytochrome P450 aromatase mRNA in granulosa cells from control women and women with polycystic ovary syndrome (PCOS). Aromatase mRNA was measured in extracts of granulosa cells isolated from 24 individual follicles from 12 women with PCOS and 33 follicles from 24 regularly cycling control women by a quantitative reverse transcription–polymerase chain reaction (RT–PCR) assay. The DNA content of the granulosa cells was measured by a sensitive fluorescent assay.

Expression of $P450_{AROM}$ mRNA in PCOS follicles

To determine if the pattern of $P450_{AROM}$ expression was altered in PCOS, a quantitative RT–PCR assay was developed to measure human $P450_{AROM}$ mRNA in granulosa cells samples from individual follicles. A typical standard curve using cloned $P450_{AROM}$ cDNA is shown in Figure 4. The assay yields a linear standard curve in the range 0.07–4.5 pg of full length $P450_{AROM}$ mRNA. The inter-assay and intra-assay coefficients of variation were both $<$16%.

As shown in Figure 5, $P450_{AROM}$ mRNA levels were very low in the granulosa cells of control follicles with androstenedione:oestradiol ratios of $>$4. Beginning at ~7 mm in diameter there was an increase in $P450_{AROM}$ mRNA in control follicles with androstenedione:oestradiol ratios of $<$4. These were the same follicles that had increased levels of oestradiol and aromatase stimulating bioactivity in the follicular fluid. In PCOS follicles the $P450_{AROM}$ mRNA in the granulosa cells remained low. The levels were similar to the levels found in granulosa cells from control follicles with androstenedione:-oestradiol ratios of $>$4 and never approached the levels found in follicles that showed increases in oestradiol values.

Discussion

The mechanism of the arrest of follicle development in PCOS has been the subject of intense interest. One of the key characteristics of dominant follicles is the large increase in aromatization of androgens by the granulosa cells that is reflected in high follicular fluid concentrations of oestradiol and an androstenedione:oestradiol ratio of $<$4 (McNatty *et al.*, 1979; Brailly *et al.*, 1981). In the follicular fluid of polycystic ovaries, oestradiol concentrations never reach the levels found in dominant follicles and the androstenedione:oestradiol ratio remains $>$4 except in the rare instance when a dominant follicle develops (Pache *et al.*, 1992). The present data as well as previous publications demonstrate that the lack of oestradiol production is clearly not due to the lack of androgen substrate (San Roman and Magoffin, 1992; Pache *et al.*, 1992; Mason *et al.*, 1994). In the present study, when follicular fluid oestradiol concentrations were analysed as a function of follicle diameter the data showed that oestradiol concentrations were low in small antral follicles and began to increase in some of the 7 mm follicles of control women. Presumably the increase in $P450_{AROM}$ expression and oestradiol production were in follicles that would have become large dominant follicles. These data are in agreement with previous studies demonstrating that follicular fluid oestradiol concentrations are elevated in follicles $>$8 mm relative to follicles $<$8 mm (Ryan and Petro, 1966; McNatty *et al.*, 1975; van Dessel *et al.*, 1996)

and that increased serum oestradiol in the follicular phase occurs when the dominant follicle grows >9 mm (van Santbrink *et al.*, 1995). In PCOS follicles, the low follicular fluid oestradiol concentrations were not different from size matched follicles in regularly cycling control women. These results are in agreement with previous studies that also found similar oestradiol concentrations (McNatty *et al.*, 1980; Pache *et al.*, 1992; Mason *et al.*, 1994). Our findings are also in agreement with the only study to address the endogenous aromatase activity of granulosa cells in PCOS (Erickson *et al.*, 1979). In short-term incubations of granulosa cells from follicles of various sizes, it was shown that little or no aromatase activity was present in granulosa cells from follicles <8 mm in diameter from either PCOS or control women.

Previously it has been unclear whether the low levels of oestradiol production in PCOS were because the granulosa cells did not express $P450_{AROM}$ or were caused by inhibition of $P450_{AROM}$ activity by endogenous inhibitors. Our studies measuring $P450_{AROM}$ mRNA expression in the granulosa cells from individual follicles showed that the follicles which have elevated oestradiol concentrations in the follicular fluid also demonstrate increased $P450_{AROM}$ mRNA expression in their granulosa cells. Follicles <7 mm in diameter contained extremely low or undetectable levels of $P450_{AROM}$ mRNA whether they were from PCOS or regularly cycling women. These data support the conclusion that PCOS follicles have low levels of oestradiol in their follicular fluid because they have little or no $P450_{AROM}$ enzyme activity (Erickson *et al.*, 1979) and that this is normal for follicles <7 mm in diameter. These data are also consistent with the concept that the transition to follicular dominance begins when the follicle grows to ~7 mm in diameter.

It has been established that >1.3 mIU/ml of immunoreactive FSH in the follicular fluid are required for a follicle to contain the elevated follicular fluid levels of oestradiol characteristic of dominant follicles (McNatty and Baird, 1978) and that immunoreactive FSH concentrations in follicular fluid increase as follicle diameter increases (McNatty *et al.*, 1975). The present data are the first to measure the aggregate aromatase stimulating bioactivity (FSH plus growth and differentiation factor net bioactivity) in the follicular fluid of individual follicles. In agreement with the immunoassay data, aromatase stimulating bioactivity was low in the follicular fluid of follicles <7 mm in diameter and in larger follicles that did not exhibit increased oestradiol concentrations. In every instance where a follicle contained oestradiol concentrations >300 ng/ml there was >0.5 mIU FSH equivalent activity/ml of aromatase stimulating bioactivity in the follicular fluid suggesting that a mechanism exists in follicles that become dominant which allows FSH and/or growth factors to accumulate in the follicular fluid. In follicles that do not become dominant it appears that FSH is excluded from the follicular fluid (McNatty *et al.*, 1975). The pattern of aromatase stimulating bioactivity in the follicular fluid of developing follicles is similar to that previously reported for immunoreactive FSH (McNatty *et al.*, 1975) suggesting that regulation of the entry of FSH into the follicular microenvironment is central to the process of follicular dominance.

In PCOS follicles, the low levels of aromatase stimulating bioactivity were similar to size matched control follicles. Our data measure the net effect of all of the bioactive substances present in the follicular fluid that influence oestradiol production including FSH as well as a variety of growth and differentiation factors such as insulin-like growth factors (Mason *et al.*, 1996) and their binding proteins (Cataldo and Giudice, 1992; San Roman and Magoffin, 1992), inhibins and activin (Magoffin and Jakimiuk, 1997), follistatin (Erickson *et al.*, 1995), epidermal growth factor (EGF) (Mason *et al.*, 1990) and tumour necrosis factor-α (TNF-α) (Rice *et al.*, 1996). As such these data reflect the physiological microenvironment and demonstrate that in follicles from polycystic ovaries the net stimulatory bioactivity is insufficient to induce the expression of $P450_{AROM}$ mRNA. A previous study showed that in follicles where FSH levels were detectable the median immunoreactive FSH levels were the same (0.3 IU/L) in the follicular fluid of PCOS follicles and follicles from ovulatory women (Mason *et al.*, 1994). The immunoreactive FSH concentrations were below the detection limit of 0.3 IU/L in more than half of the follicular fluids measured. The present results are consistent with these findings. Another study measured ⩾5 mIU/ml of bioactive FSH concentration in pooled follicular fluid from three women with PCOS (Erickson *et al.*, 1992). These bioactivity data appear to be inconsistent with the reported immunoassay data demonstrating that FSH concentrations were very low in most PCOS follicles (Mason *et al.*, 1994). In at least one follicle, however, the immunoreactive FSH concentration was found to be 3.8 IU/L (Mason *et al.*, 1994), indicating that FSH does accumulate in a very few PCOS follicles. Our data do not isolate the contributions of individual hormones to the net bioactivity. If the FSH bioactivity is high in PCOS as was previously reported, our data indicate that the follicular fluid contains substances that block the stimulatory actions of the FSH.

One of the keys to the progression of dominant follicle development is the transition from FSH-independent growth to FSH-dependent development. In PCOS the great majority of developing follicles do not make the transition to FSH-dependent development. This is not due to an inherent defect in the granulosa cells because the granulosa cells are actually more sensitive to FSH *in vitro* than granulosa cells from ovulatory women (Erickson *et al.*, 1992; Mason *et al.*, 1994) and because administration of exogenous FSH can stimulate preovulatory follicle development in women with PCOS (Fauser, 1994). Our observation that a small proportion of PCOS follicles begin to have elevated aromatase stimulating bioactivity but do not appear to respond to the FSH is consistent with the concept that PCOS follicles contain inhibitors of FSH action. Several molecules have been suggested as potential inhibitors including a high molecular weight FSH receptor binding inhibitor (Lee *et al.*, 1993), IGFBP (Ui *et al.*, 1989), EGF (Mason *et al.*, 1990) and TNF-α (Rice *et al.*, 1996). There do not appear to be alterations in the concentrations of FSH receptor inhibitory activity in PCOS women relative to regularly cycling women (Schipper *et al.*, 1997). It remains to be proven if the physiological concentrations of any of the other potential inhibitors in the follicular fluid are altered in

polycystic ovaries and if the alterations are significant enough to block follicle development. Recently, we have shown that 5α-androstane-3, 17-dione, a competitive inhibitor or $P450_{AROM}$ activity, is present in markedly elevated concentrations in PCOS follicular fluid that are sufficient to maximally block $P450_{AROM}$ activity in the presence of physiological concentrations of androgen substrates (Agarwal *et al.*, 1996). In light of the present data that $P450_{AROM}$ expression is low in normal follicles <7 mm, it is unclear whether 5α-androstane-3, 17-dione plays an important role as an inhibitor of $P450_{AROM}$ activity in PCOS or if 5α-androstane-3, 17-dione may interfere with follicle development by mechanisms involving an androgen receptor-mediated mechanism. It is possible that inhibition of $P450_{AROM}$ activity when $P450_{AROM}$ is first expressed may prevent important increases in oestradiol production that could be important for dominant follicle development. Acting indirectly through suppression of oestradiol concentrations or directly through its androgenic activity, 5α-androstane-3, 17-dione may also inhibit the expression of $P450_{AROM}$ mRNA in the granulosa cells. Further studies will address these possibilities.

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References

Agarwal, S.K., Judd, H.L. and Magoffin, D.A. (1996) A mechanism for the suppression of estrogen production in polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.*, **81,** 3686–3691.

Brailly, S., Gougeon, A., Milgrom, E. *et al.* (1981) Androgens and progestins in the human ovarian follicle: differences in the evolution of preovulatory, healthy nonovulatory, and atretic follicles. *J. Clin. Endocrinol. Metab.*, **53,** 128–134.

Cataldo, N.A. and Giudice, L.C. (1992) Insulin-like growth factor binding protein profiles in human ovarian follicular fluid correlate with follicular functional status. *J. Clin. Endocrinol. Metab.*, **74,** 821–829.

Corbin, C.J., Graham-Lorence, S., McPhaul, M. *et al.* (1988) Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. *Proc. Natl. Acad. Sci. USA*, **85,** 8948–8952.

Downs, T.R. and Wilfinger, W.W. (1983) Fluorometric quantification of DNA in cells and tissues. *Anal. Biochem.*, **131,** 538–547.

Erickson, G.F. and Yen, S.S.C. (1984) New data on follicle cells in polycystic ovaries: a proposed mechanism for the genesis of cystic follicles. *Sem. Reprod. Endocrinol.*, **42,** 39–43.

Erickson, G.F., Hsueh, A.J.W., Quigley, M.E. *et al.* (1979) Functional studies of aromatase activity in human granulosa cells from normal and polycystic ovaries. *J. Clin. Endocrinol. Metab.*, **49,** 514–519.

Erickson, G.F., Magoffin, D.A., Garzo, V.G. *et al.* (1992) Granulosa cells of polycystic ovaries: are they normal or abnormal? *Human Reprod.*, **7,** 293–299.

Erickson, G.F., Chung, D., Sit, A. *et al.* (1995) Follistatin concentrations in follicular fluid of normal and polycystic ovaries. *Human Reprod.*, **10,** 2120–2124.

Fauser, B.C.J.M. (1994) Step-down follicle-stimulating hormone regimens in polycystic ovary syndrome. In Filicori, M. and Flamigni, C.(eds), *Ovulation Induction: Basic Sciences and Clinical Advances.* Elsevier Science B.V. Amsterdam, p. 153.

Goldzieher, J.W. (1981) Polycystic ovarian disease. *Fertil. Steril.*, **35,** 371–394.

Goldzieher, J.W., Axelrod, L.R. (1963) Clinical and biochemical features of polycystic ovarian disease. *Fertil. Steril.*, **14,** 631–653.

Gougeon, A. (1986) Dynamics of follicular growth in the human: a model from preliminary results. *Hum. Reprod.*, **2,** 81–87.

Horton, R.M., Cai, Z., Ho, S.N. and Pease, L.R. (1990) Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques*, **8,** 528–534.

Jia, X., Kessel, B., Yen, S.S.C. *et al.* (1986) Serum bioactive follicle-stimulating hormone during the human menstrual cycle and in hyper- and hypogonadotropic states: application of a sensitive granulosa cell aromatase bioassay. *J. Clin. Endocrinol. Metab.*, **62,** 1243–1249.

Lee, D.W., Grasso, P., Dattatreyamurty, B. *et al.* (1993) Purification of a high molecular weight follicle-stimulating hormone receptor-binding inhibitor from human follicular fluid. *J. Clin. Endocrinol. Metab.*, **77,** 163–168.

Lobo, R.A. (1995) A disorder without identity: 'HCA,' 'PCO,' 'PCOD,' 'PCOS,' 'SLS'. What are we to call it?! *Fertil. Steril.*, **63,** 1158–1160.

Magoffin, D.A., Erickson, G.F. (1982) Primary culture of differentiating ovarian androgen-producing cells in defined medium. *J. Biol. Chem.*, **257,** 4507–4513.

Magoffin, D.A. and Jakimiuk, A.J. (1997) Inhibin A, inhibin B and activin A in the follicular fluid of regularly cycling women. *Hum. Reprod.*, **12,** 1714–1719.

Mason, H.D., Margara, R., Winston, R.M.L. *et al.* (1990) Inhibition of oestradiol production by epidermal growth factor in human granulosa cells of normal and polycystic ovaries. *Clin. Endocrinol.*, **33,** 511–517.

Mason, H.D., Willis, D.S., Beard, R.W. *et al.* (1994) Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentraions of gonadotropins and sex steroids in follicular fluid. *J. Clin. Endocrinol. Metab.*, **79,** 1355–1360.

Mason, H.D., Cwyfan-Hughes, S.C., Heinrich, G. *et al.* (1996) Insulin-like growth factor (IGF) I and II, IGF-binding proteins, and IGF-binding protein proteases are produced by theca and stroma of normal and polycystic human ovaries. *J. Clin. Endocrinol. Metab.*, **81,** 276–284.

McNatty, K.P. and Baird, D.T. (1978) Relationship between follicle-stimulating hormone, androstenedione and oestradiol in human follicular fluid. *J. Endocrinol.*, **76,** 527–531.

McNatty, K.P., Hunter, W.M., McNeilly, A.S. and Sawers, R.S. (1975) Changes in the concentration of pituitary and steroid hormones in the follicular fluid of human graafian follicles throughout the menstrual cycle. *J. Endocrinol.*, **64,** 555–571.

McNatty, K.P., Smith, D.M., Makris, A. *et al.* (1979) The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte *in vivo* and *in vitro*. *J. Clin. Endocrinol. Metab.*, **49,** 851–860.

McNatty, K.P., Smith, D.M., Makris, A. *et al.* (1980) The intraovarian sites of androgen and estrogen formation in women with normal and hyperandrogenic ovaries as judged by *in vitro* experiments. *J. Clin. Endocrinol. Metab.*, **50,** 755–763.

Pache, T.D., Hop, W.C.J., De Jong, F.H. *et al.* (1992) 17β-oestradiol, androstenedione and inhibin levels in fluid from individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals. *Clin. Endocrinol.*, **36,** 565–571.

Polson, D.W., Adams, J., Wadsworth, J. and Franks, S. (1988) Polycystic ovaries – a common finding in normal women. *Lancet*, **i,** 870–872.

Rice, V.M., Williams, V.R., Limback, S.D. and Terranova, P.F. (1996) Tumour necrosis factor-alpha inhibits follicle stimulating hormone-induced granulosa cell oestradiol secretion in the human: dependence on size of follicle. *Hum. Reprod.*, **11,** 1256–1261.

Ryan, K.J. and Petro, Z. (1966) Steroid biosynthesis by human ovarian granulosa and thecal cells. *J. Clin. Endocrinol. Metab.*, **26,** 46–52.

San Roman, G.A. and Magoffin, D.A. (1992) Insulin-like growth factor binding proteins in ovarian follicles from women with polycystic ovarian disease: cellular source and levels in follicular fluid. *J. Clin. Endocrinol. Metab.*, **75,** 1010–1016.

Schipper, I., Rommerts, F.F.G., Ten Hacken, P.M. and Fauser, B.C.J.M. (1997) Low levels of follicle-stimulating hormone receptor-activation inhibitors in serum and follicular fluid from normal controls and anovulatory patients with or without polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.*, **82,** 1325–1331.

Schneyer, A.L., Sluss, P.M., Whitcomb, R.W. *et al.* (1991) Precursors of α-inhibin modulate folicle-stimulating hormone receptor binding and biological activity. *Endocrinology*, **129,** 1987–1999.

Ui, M., Shimonaka, M., Shimasaki, S. and Ling, N. (1989) An insulin-like growth factor-binding protein in ovarian follicular fluid blocks follicle-stimulating hormone-stimulated steroid production by ovarian granulosa cells. *Endocrinology,* **125,** 912–916.

van Dessel, T.J.H.M., Schipper, I., Pache, T.D. *et al.* (1996) Normal human follcile development: an evaluation of correlations with oestradiol, androstenedione and progesterone levels in individual follicles. *Clin. Endocrinol.,* **44,** 191–198.

van Santbrink, E.J.P., Hop, W.C., van Dessel, T.J.H.M. *et al.* (1995) Decremental follicle-stimulating hormone and dominant follicle development during the normal menstrual cycle. *Fertil. Steril.,* **64,** 37–43.

Yen, S.S.C. (1980) The polycystic ovary syndrome. *Clin. Endocrinol.*, **12,** 177–208.

Zeleznik, A.J., Fairchild-Benyo, D. (1994) Control of follicular development, corpus luteum function, and the recognition of pregnancy in higher primates. In Knobil, E. and Neill, J.D.(eds), *The Physiology of Reproduction.* Raven Press Ltd, New York, p. 751.

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