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Functional, long-term human theca and granulosa cell cultures from polycystic ovaries

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Reproducible culture conditions for obtaining large numbers of functional PCOS theca interna and granulosa-lutein cells will be indispensable in studies focussing on the molecular basis for androgen overproduction by ovarian cells of patients with polycystic ovarian syndrome (PCOS). The objective of the present study was to determine if granulosa and theca interna cells obtained from ovarian follicles of patients with PCOS could be passaged with maintenance of inducible steroidogenic activity. PCOS theca interna and granulosa cells were obtained from individual follicles of polycystic ovaries containing multiple cystic follicles with characteristic hypertrophied theca interna. Utilizing conditions for growing normal ovarian cells, both cell types were passaged successively and conditions for cell freezing, storing and thawing were established. In granulosa-lutein cultures grown and passed for successive passages, and transferred into serum-free medium, forskolin stimulated aromatase activity increased 3–10-fold over control non-stimulated values. Concurrent treatment with IGF-I (50 ng/mL) enhanced forskolin-stimulated aromatase activity in PCOS granulosa-lutein cultures. In passaged PCOS theca interna cells, forskolin-stimulated 17α-hydroxyprogesterone production was increased 4–25-fold over control values. Treatment of PCOS theca interna cells with insulin (50 ng/mL) enhanced forskolin-stimulated 17α-hydroxyprogesterone biosynthesis. The effects of various growth factors and phorbol esters on 17α-hydroxylase activity in cultured PCOS theca interna cells was also investigated. Treatment of PCOS theca cells with EGF, FGF, TGFβ and TPA resulted in the inhibition of forskolin-stimulated 17α-hydroxyprogesterone production. These data suggest that PCOS theca interna and granulosa cells respond to insulin and to the growth factors similarly to cells obtained from normal cycling ovaries.

Keywords: polycystic ovary; theca; granulosa; human ovary

Introduction

PCOS is generally recognized as the most common cause of infertility in reproductive age women. Reproductive abnormalities include ovarian cysts, irregular menstrual cycles, infertility and hirsutism or virilism of various degrees resulting from increased ovarian androgen production (Yen, 1980; Goldzieher, 1981; Franks, 1989). Polycystic ovaries are characterized by large numbers of small follicles (4–7 mm in diameter) with an hypertrophied theca interna layer (Goldzieher & Green, 1962; Erickson *et al.*, 1985; Franks, 1992). Multiple pathogenic mechanisms have been proposed suggesting that the abnormality resulting in polycystic ovarian syndrome may arise from defects in the hypothalamic-pituitary axis, ovary or adrenal (Yen, 1980; Goldzieher, 1981; Barbieri *et al.*, 1988a,b; Franks *et al.*, 1988). However, in many cases no specific cause for polycystic ovaries or hyperandrogenism has been identified.

There have been a variety of reports on the regulation of steroid production by ovarian cells isolated from ovaries of patients with polycystic ovarian disease. A number of these studies have predominately focussed on the regulation of estrogen production in primary granulosa cells from PCOS patients (Erickson *et al.*, 1979; Wilson *et al.*, 1979; Erickson *et al.*, 1990, Mason *et al.*, 1991) and only a handful of studies have focussed on the androgen production by theca interna cells (Barbieri *et al.*, 1986; Barbieri *et al.*, 1988a,b; Erickson *et al.*, 1989). These studies have provided extremely valuable information, however limitations in the number of cells obtained from a PCOS ovary has restricted further studies requiring large numbers of PCOS cells. We have previously reported conditions for propagating large numbers of theca interna and granulosa-lutein cells from follicles of ovaries obtained from normal cycling women (McAllister *et al.*, 1989, McAllister *et al.*, 1990). Utilizing these culture conditions, we have been able to passage theca interna and granulosa lutein cells from ovaries obtained from women with PCOS. Conditions for growing, passaging, freezing, thawing and storing both granulosa-lutein cells and theca interna cells isolated from individual PCOS follicles have also been determined. Frozen cell stocks from an individual follicle can be conveniently thawed and numerous comparisons can be made between follicles obtained from a single patient. Utilizing these conditions, the steroidogenic capacity in addition to other biochemical parameters can be compared in theca and granulosa cells isolated from the same follicle. In this report we have begun to characterize the steroidogenic capacity of PCOS granulosa-lutein and theca cells in long-term culture by investigating the effects of cyclic AMP and growth factors on ovarian estrogen and androgen production. In granulosa-lutein cell cultures we investigated the regulation of the enzyme cytochrome P450 aromatase ($P450_{AROM}$), a key enzyme essential in the conversion of thecal derived androgens to estrogens. The enzyme cytochrome P450 17α-hydroxylase ($P450_{17\alpha}$), is a key enzyme involved in the production of the theca derived androgenic steroids (androstenedione and dehydroepiandrosterone), and their precursors (17α-hydroxyprogesterone and 17α-

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hydroxypregnenolone). In normal human theca interna cells in long-term culture we have previously characterized the regulation of 17α-hydroxylase activity and 17α-hydroxyprogesterone production in response to cAMP and various growth factors (McAllister *et al.*, 1989, McAllister & Simpson, 1993, 1994). In these studies, the close correlation of 17α-hydroxylase activity with 17α-hydroxyprogesterone production was determined. Hence, to screen for the maintenance of inducible 17α-hydroxylase activity in long-term PCOS theca interna cultures we examined 17α-hydroxyprogesterone production.

Results

Morphology of the PCOS ovaries

Grossly the ovaries were large and round, with multiple cystic follicles. As indicated by the arrows in Figure 1, representative microscopic sections from one ovary revealed the presence of numerous cystic follicles (3–7 mm). Closer examination (82.5 ×) showed that a representative single follicle was lined by non-luteinized granulosa cells, an outer, thicker layer of theca cells, and a thickened fibrotic vascular stroma (Figure 2), which are characteristic of polycystic ovarian syndrome (Franks, 1992).

Aromatase activity in human granulosa-lutein cells: Effects of forskolin, insulin and IGF-I

To investigate the effects of insulin and IGF-I on aromatase activity in fourth passage PCOS granulosa-lutein cells, granulosa-lutein cells were grown and transferred into serum-free medium containing no treatment (C) or 7.5 μM forskolin treatment (F), in the presence of 50 ng/ml insulin or 50 ng/ml IGF-I. 48 h following treatment, the medium was removed and aromatase activity was assayed. The data presented is representative of data obtained from screening granulosa cells isolated from eight different follicles from a single patient. In long-term cultures of granulosa-lutein cells isolated from normal cycling ovaries, we have previously reported that granulosa-lutein cells round up and pull away from the dish (retract), unlike theca interna cells, in response to forskolin treatment (McAllister *et al.*, 1989). In these experiments, following visual inspection ~100% of the PCOS granulosa cells retracted in response to forskolin treatment, indicating that the cultures were relatively pure granulosa-lutein cell cultures, free from contaminating theca cells or fibroblasts. As shown in Figure 3, in granulosa-lutein cell propagated from three follicles (A, B and C), aromatase activity was increased in response to forskolin in the absence of insulin or IGF-I. Aromatase activity in the absence of forskolin was not affected by treatment with insulin or IGF-I. Concurrent treatment of granulosa-lutein cells with forskolin and insulin resulted in a slight, but significant increase in aromatase activity as compared to forskolin treatment alone in two of the three follicles (A and B). Treatment of granulosa-lutein cells with IGF-I and forskolin resulted in an increase in aromatase as compared to treatment with forskolin alone ($P<0.05$). Aromatase activity in cells treated with the combination of IGF-I plus forskolin was also greater than that observed following treatment with insulin plus forskolin. This data confirms that aromatase activity can be induced in PCOS derived granulosa-lutein cells propagated in culture, and has been verified using cells from other patients. In addition, it appears that IGF-I, through the type I IGF receptor, modulates forskolin-stimulated aromatase activity.

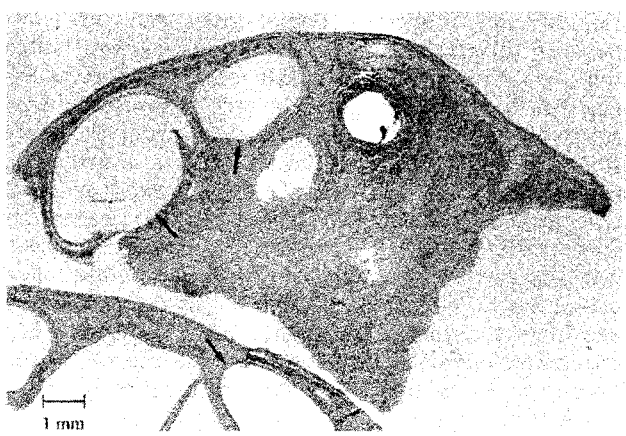

Figure 1 Cross section (10 ×) of a characteristic PCOS ovary showing multiple follicles as indicated by arrows of approximately 3–6 mm diameter

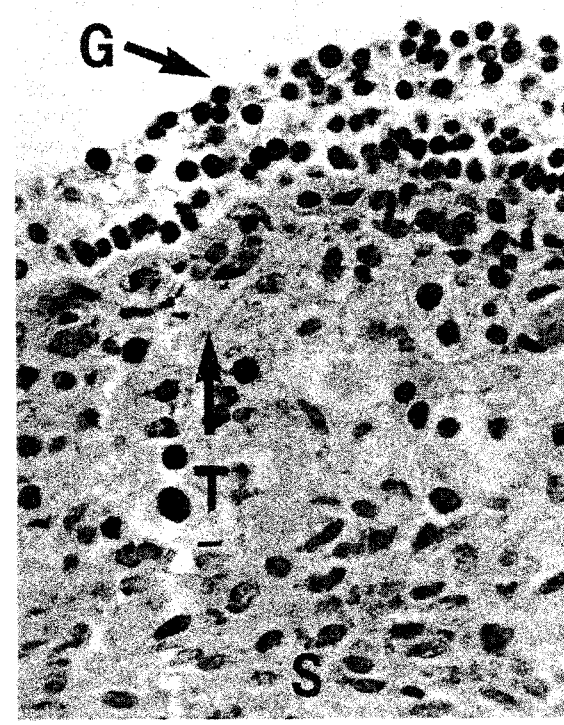

Figure 2 Magnified section (82.5 ×) of a single cystic follicle lined with non-luteinized granulosa cells (G) and an outer thickened layer of hypertrophied theca interna cells (T) surrounded by stromal cells (S)

17α-hydroxyprogesterone production in passaged PCOS theca interna cells: Effects of forskolin, insulin and IGF-I

Theca interna cells isolated from eight individual follicles were propagated, and 17α-hydroxyprogesterone production was investigated in response to treatment with forskolin, an activator of adenylate cyclase. In Figure 4, we present data from fourth passage PCOS theca interna cells, isolated from follicles A, B and C that correspond to the same follicles from which granulosa cells were obtained as shown in Figure 3. PCOS theca interna cells were grown and transferred into serum-free medium containing no treatment (C) or 7.5 μM forskolin treatment (F), in the presence of 50 ng/ml insulin or 50 ng/ml IGF-I. Seventy-two hours following treatment, the medium was collected and 17α-hydroxyprogesterone production was determined by RIA. These long-term PCOS theca interna cultures appeared to be morphologically similar to theca interna cells propagated from normal cycling ovaries. Theca interna cells propagated from normal cycling ovaries do not retract in response to stimulation with forskolin (McAllister *et al.*, 1989). Following treatment with forskolin PCOS theca interna cells appeared to be relatively pure without significant granulosa cell contamination as determined by visual inspection using an inverted phase contrast microscope following treatment with forskolin.

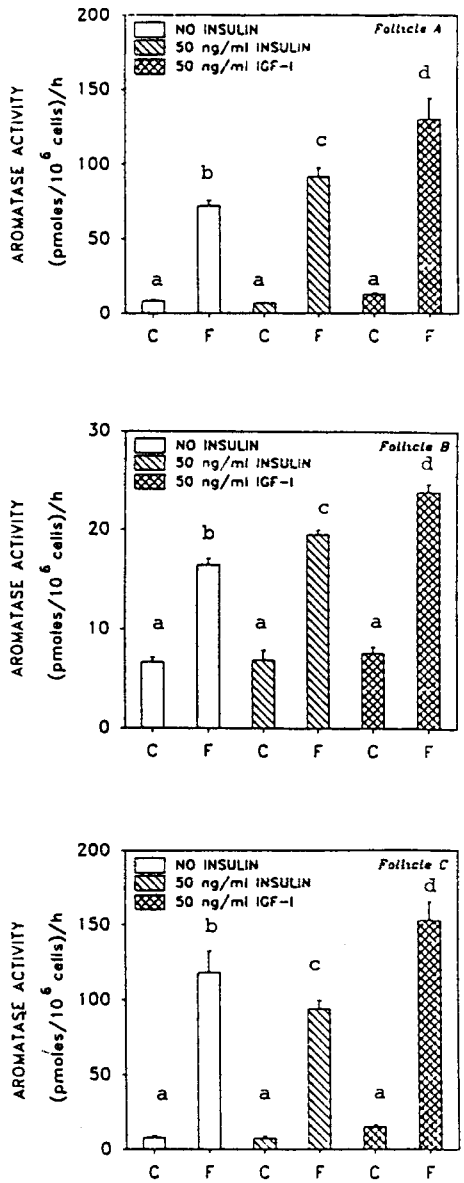

Figure 3 Aromatase activity in fourth passage PCOS granulosa-lutein cells from three individual follicles, maintained in serum-free medium in response to 48 h without treatment (C), treatment with insulin (50 ng/mL) or IGF-I (50 ng/mL) in the presence or absence of 7.5 μmol/L forskolin (F). Each data point represents the mean ± SEM of results derived from four replicates dishes. Values with differing superscripts differed ($P<0.05$)

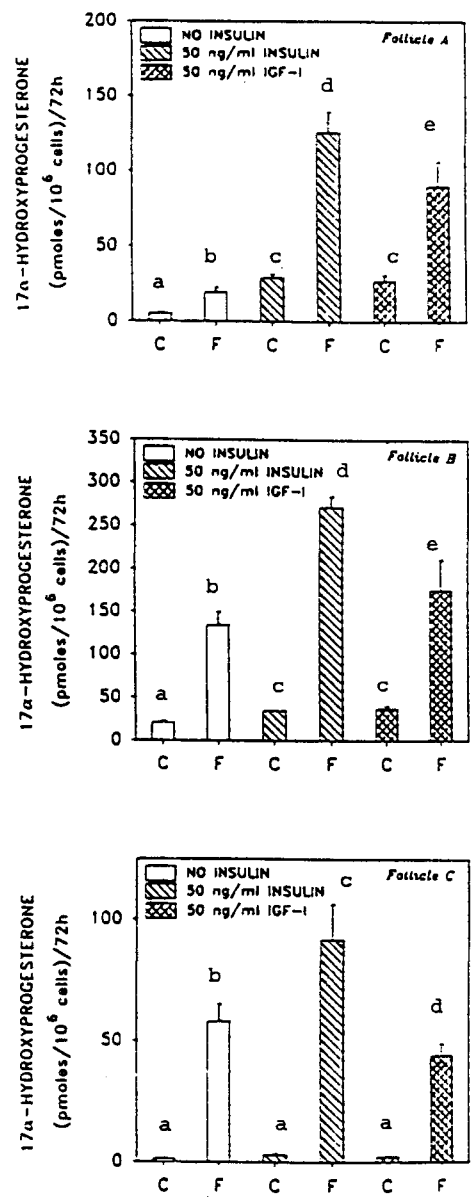

Figure 4 17α-Hydroxyprogesterone synthesis by fourth passage PCOS theca interna cells isolated from three individual follicles, maintained in serum-free medium in response to no treatment (C), treatment with insulin (50 ng/mL) or IGF-I (50 ng/mL) in the presence or absence of 7.5 μmol/L forskolin (F). Following 72 h treatment the medium was collected and 17α-hydroxyprogesterone accumulation was estimated by RIA. Cell number was determined for each dish after trypsinization using a Coulter Counter. Each data point represents the mean ± SEM of results derived from four replicate dishes. Values with differing superscripts differed ($P<0.05$)

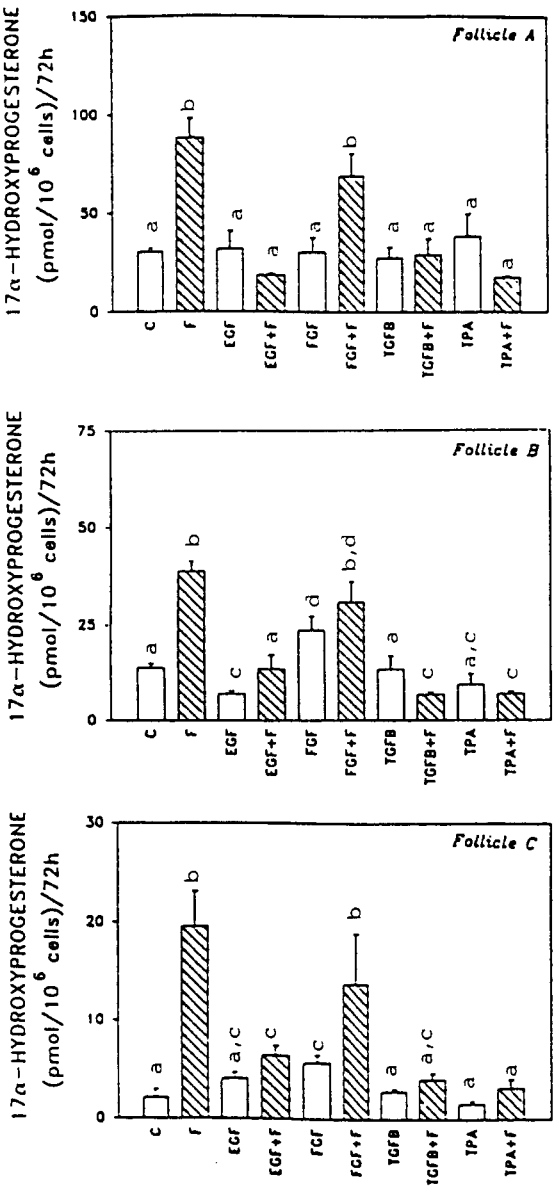

Figure 5 Effects of growth factors on forskolin-stimulated 17α-hydroxyprogesterone accumulation in theca interna cells isolated from 3 individual follicles. 17α-hydroxyprogesterone accumulation into the medium of fourth passage theca interna cells following 72 h with no treatment (C), or treatment with EGF (50 ng/mL), FGF (100 ng/mL), TGFβ (5.0 ng/mL) or TPA (10 nmol/L), alone or in the presence of forskolin (7.5 μmol/L). Each data point represents the mean ± SEM of results derived from four replicates dishes. Values with differing superscripts differed ($P<0.05$)

In long-term cultures of PCOS theca interna cells, treatment with forskolin resulted in a marked stimulation of 17α-hydroxyprogesterone production (Figure 4). In the absence of forskolin, 17α-hydroxyprogesterone production was only slightly stimulated by insulin or IGF-I in two of the three follicles (A and B) while in follicle C it was not. Treatment with insulin significantly increased forskolin stimulated 17α-hydroxyprogesterone production in all follicles. In contrast, treatment with IGF-I plus forskolin also significantly stimulated 17α-hydroxyprogesterone production to a level less than for insulin and forskolin.

Effect of EGF, FGF, TGFβ and TPA treatment on forskolin stimulated 17α-hydroxyprogesterone production in PCOS theca interna cells

To investigate the effects of growth factor treatment on androgen production, 17α-hydroxyprogesterone accumulation into the medium was measured in theca interna cells from follicles A, B and C, treated with maximally effective concentrations of EGF (50 ng/mL), FGF (200 ng/mL), TGFβ (5.0 ng/mL) and TPA (10 nmol/L) alone or in the presence of 7.5 μM forskolin (Figure 5). Treatment of theca interna cells with EGF, TGFβ and TPA had no effect alone, but resulted in the complete inhibition of forskolin-stimulated 17α-hydroxylase activity ($P<0.05$). Treatment with FGF, did not significantly inhibit forskolin stimulated 17α-hydroxyprogesterone production ($P<0.05$).

Discussion

Using culture conditions developed for normal theca interna and granulosa-lutein cells, we have recently begun to passage theca interna cells from ovaries obtained from patients with PCOS. The ability to passage and stock PCOS granulosa-lutein and theca interna cells from individual follicles provides a reproducible system for obtaining a large number of functional cells to investigate a number of biochemical parameters and to compare various parameters from granulosa or theca cells obtained from the same follicle. It also provides a means to determine if there are intrinsic defects in granulosa-lutein and theca interna cells obtained from PCOS patients that result in abnormal follicle development and ovarian hyperandrogenism.

A number of different mechanisms have been proposed in an attempt to explain the etiology of PCOS (Goldzieher *et al.*, 1962; Yen, 1980; Goldzieher, 1981; Franks, 1989; Franks, 1992). PCOS is characterized by a disruption in ovarian steroid production and chronic anovulation. It appears that in the initial stages of folliculogenesis, follicle recruitment and growth are normal in patients with PCOS (Erickson *et al.*, 1989). However, selection of the dominant follicle or preovulatory follicle appears to be defective, and as a result there is an accumulation of numerous small follicles (4–7 mm) in the ovaries of patients with PCOS. PCOS follicles also have characteristic histologic features which include a moderately undifferentiated granulosa cell layer lining and a hypertrophied theca interna layer (Figure 1 and 2).

The PCOS ovary has been shown to produce extremely high levels of the 17α-hydroxylated androgens (i.e., androstenedione and dehydroepiandrosterone) (Barbieri *et al.*, 1988a). In contrast, to the abnormally high level of androgens, the PCOS ovary has been shown to produce reduced levels of estradiol in comparison to normal ovaries. It has been demonstrated that aromatase activity is deficient in ovaries of patients with PCOS (Axelrod & Goldzieher, 1961; Mahesh & Greenblat, 1964; Erickson *et al.*, 1979; Goldzieher, 1981). As a result of these studies it was proposed that androgen production was high as a consequence of deficient granulosa cell associated aromatase activity and the inability of granulosa cells

to convert androgens to estrogens. However Erickson *et al.* (1979), has convincingly demonstrated that PCOS granulosa cells in primary culture produce estradiol (E_2) in response to FSH treatment. Hence, PCOS granulosa cells *in vitro* have inducible aromatase activity. Moreover, these authors reported that FSH stimulated E_2 production by PCOS granulosa cells in primary culture was enhanced by IGF-I (10–100 ng/mL). In long-term, passaged PCOS granulosa-lutein cells (Figure 3), aromatase activity was also shown to be induced following treatment with forskolin. In addition, we have found a similar enhancement of forskolin stimulated aromatase activity with IGF-I (Figure 3). Hence it appears that our long-term cultures respond similarly to primary cells as described by Erickson *et al* (1979, 1992).

We have previously reported (McAllister, 1989) that granulosa cells from normal cycling ovaries are morphologically distinct from theca interna cells following treatment with forskolin. Granulosa cells retract and round up in response to activators of adenylate cyclase, whereas theca interna cells do not retract. Hence, theca culture contamination with granulosa cells, or granulosa culture contamination with theca cells, can easily be assessed by observing the morphological changes which occur in long-term granulosa and theca cell culture in response to forskolin. PCOS granulosa-lutein cells were observed to retract and rounded up in response to forskolin treatment. These observations are in agreement with the morphological responses of normal theca interna and granulosa-lutein cells under similar conditions (McAllister *et al.*, 1989, 1990). Theca interna cells used in these studies were relatively pure, in that they do not contain contaminating granulosa cells. In contrast, granulosa cultures, did not appear to contain non retracting theca interna or fibroblasts. In addition, long-term cultures of PCOS theca grown in these studies were found to be relatively free from granulosa-lutein cells.

As shown in Figure 4, PCOS theca interna cells in long-term culture are highly sensitive to forskolin, an activator of cAMP. These results are in agreement with studies by Barbieri *et al.* (1986), who investigated the regulation of androgen synthesis in isolated theca interna cells from PCOS ovaries. Barbieri *et al.* (1986, 1988a) investigated the effects of LH and insulin on testosterone release in minced ovarian stroma. Data from these studies indicated that ovarian stromal cells from PCOS patients are exquisitely sensitive to LH and insulin. In addition these data, and studies from Erickson *et al.* (1989) have confirmed that there is abnormal regulation of androgen production in the ovaries of PCOS patients, irrespective of high circulating bioactive levels of LH. Hence, the ability to propagate PCOS theca interna cells provides a system to further study the regulation of androgen production by PCOS theca interna cells.

In the last several years it has been proposed that insulin resistance and hyperinsulinemia in PCOS can somehow cause increased androgen production by theca interna cells of the ovary (Barbieri *et al.*, 1988). It has been suggested that insulin acts through spill-over occupancy of the IGF-I receptor leading to the stimulation of ovarian androgen production. However our preliminary studies with normal and PCOS theca interna cells suggest that insulin may act via its own receptor, rather than through spill-over occupancy of the IGF-I receptor (Figure 4), in the enhancement of forskolin stimulated 17α-hydroxyprogesterone production.

It has been proposed that normal follicle development and selection during the menstrual cycle is regulated via the combined actions of gonadotrophins and intra-ovarian growth factors. It has also been proposed that the regulation of expression of the steroidogenic enzymes throughout the human ovarian cycle results from the differential regulation of these enzymes by both gonadotrophins and intra-ovarian growth factors (McAllister *et al.*, 1994). In the PCOS ovary, follicle development, recruitment and steroid production appear to be aberrant. Hence, it is possible that the cellular responses of PCOS theca interna cells to 'ovarian' growth factors proposed to regulate normal ovarian follicular growth and steroidogenic enzyme expression could be aberrant in PCOS cells.

In theca interna cells propagated from PCOS follicles we have found that cyclic AMP and growth factors differentially regulate 17α-hydroxylase expression, in a fashion that identically mirrors growth factor action in normal theca interna cells in culture (McAllister *et al.*, 1994). In long-term human theca interna cells isolated from normal cycling ovaries, treatment with cyclic AMP, insulin and IGF-I stimulated 17α-hydroxyprogesterone production whereas stimulation of the C-kinase pathway by phorbol esters or treatment with FGF or TGFβ, inhibited cAMP-stimulated 17α-hydroxyprogesterone production. Hence, it appears that the signal transduction pathways responsible for growth factor responsiveness are intact in PCOS theca interna, and the pathogenesis of excessive androgen production in the common disorder of ovarian hyperandrogenism or polycystic ovarian syndrome (PCOS) can not be a result of a lack of responsiveness to the growth factors studied.

In conclusion, we have presented conditions for growing functional cultures of PCOS theca interna and granulosa cells. The ability to propagate large numbers of PCOS theca interna and granulosa cells provides a means to investigate if there is an intrinsic molecular or biochemical mechanism in PCOS ovarian cells which results in aberrant follicle development and gross ovarian hyperandrogenism.

Materials and methods

Preparation of human PCOS theca interna and granulosa-lutein cells

Human theca interna tissue was obtained from preovulatory follicles of women undergoing hysterectomy. Whole individual follicles were dissected away from the ovarian stroma. The size of each follicle was recorded, and granulosa cells were isolated from each individual follicle. Primary theca interna cells and granulosa cells were obtained from each individual follicle as follows. In serum containing medium, the follicle was cut in half. Under a dissecting microscope the granulosa cells were gently brushed from the theca with a platinum loop. The granulosa cells from each individual follicle were harvested from the medium, following centrifugation at 600*g*. The theca isolated from an individual was transferred to a fresh petri dish in serum containing medium, the theca interna was stripped from the follicle wall, and a suspension of thecal cells obtained following dispersal with

0.05% collagenase I, 0.05% collagenase IA and 0.01% deoxyribonuclease in medium containing 10% fetal bovine serum (FBS) (McAllister *et al.*, 1989). Harvested granulosa cells and dispersed theca interna cells were placed in culture dishes that had been coated with fibronectin by incubation at 37°C with culture medium containing 5 μg/mL human fibronectin, which was a generous gift of Dr Peter Hornsby, Medical College of Georgia. The media used for cell plating of theca interna cells was a 1:1 mixture of Dulbecco's Eagles Medium (DME) and Hams F-12 medium containing 10% FBS, 10% horse serum (HS), 2% UltroSer G, 20 nmol/L insulin, 20 nmol/L selenium, 1 μmol/L vitamin E and antibiotics (McAllister *et al.*, 1989). Human granulosa-lutein cells were cultured in medium containing the additives described above for plating theca interna cells, however the horse and fetal bovine serums are reduced to 5% as described previously (McAllister *et al.*, 1990). The granulosa cells and theca cells from an individual follicle are labelled for future comparison. From each follicle 12–35 mm dishes of primary theca interna or granulosa cells were grown until confluent, removed from the dish with neutral protease, frozen and stored in liquid nitrogen (one 35 mm dish per vial) as previously described (McAllister *et al.*, 1989; McAllister *et al.*, 1990) in culture medium that contained 20% FBS and 10% dimethyl sulfoxide. To obtain successive passages of PCOS theca interna or granulosa cells, the cells were subcultured at a 1:6, and frozen down at each successive passage. Multiple follicles have been isolated from each ovarian specimen. At present we are capable of propagating approximately 8×10^9 functional cells from a single follicle. In addition, frozen vials of early passaged cells provides a convenient mechanism for designing experiments.

Sera and growth factors were obtained as follows: FBS was obtained from Irvine Scientific (Irvine, CA): HS was obtained from Gibco (Grand Island, N.Y.); UltroSer G was from Reactifs IBF (Villeneuve-la-Garenne, France): other compounds were from Sigma Chemical Co. (St Louis, MO). Recombinant human IGF-I was from GroPep (Adelaide, Australia), mouse EGF and human TGFβ were from AMGen (Thousand Oaks, CA) and bovine FGF was from Collaborative Research (Bedford, MA). In all experiments the gas phase used was 5% O_2, 90% N_2, and 5% CO_2. Subculture was performed by incubation with neutral protease (pronase-E;protease type XXIV, Sigma) in DME-F12 (1:1).

Histology

Ovarian tissue was formalin fixed, paraffin embedded, processed and stained with hematoxylin and eosin (Sheehan & Hrapchak, 1980).

Assay for aromatase activity in granulosa cells

Granulosa-lutein cells were grown as previously reported (McAllister *et al.*, 1990). At subconfluency, enzyme induction was investigated in defined medium as previously reported (McAllister *et al.*, 1989) in the presence or absence of forskolin and insulin or IGF-1. We have previously reported that aromatase activity is maximal following 48 h stimulation with forskolin (McAllister *et al*, 1990). Aromatase activity was assessed 48 h following treatment, by measurement of [^{3}H] water produced by the stereospecific release of tritium from [1-^{3}H]androstenedione, as previously described (Simpson *et al.*, 1981) and expressed as picomole of [^{3}H]androstenedione metabolized per 10^6 cells/h as previously reported (McAllister *et al.*, 1990). This assay was performed using optimal conditions with saturating substrate concentrations within a linear time frame, in which less than 10% of the substrate was converted to product (McAllister *et al.*, 1990).

Determination of 17α-hydroxyprogesterone production by theca interna cells

Human theca interna cells were plated on human fibronectin-coated 12- well multiwell dishes in medium containing 10% FBS, 10% HS, 2% UltroSer G, 20 nmol/L selenium, 1 μmol/L Vitamin E and antibiotics. At subconfluence, steroid production was investigated in defined medium as previously reported (McAllister *et al.*, 1989). The defined medium was comprised of DME-F12 (1:1), 1 mg/mL BSA, 100 ng/mL transferrin, 20 nmol/L selenium, 1 μmol/L vitamin E, and antibiotics (McAllister *et al.*, 1989). The medium was harvested, and the cells were trypsinized for estimation of cell number using a Coulter counter. RIAs for 17α-hydroxyprogesterone was performed without organic solvent extraction using radioimmunoassay reagents purchased from ICN Biomedical Inc. (Costa Mesa, CA.).

We have previously reported that 17α-hydroxyprogesterone accumulation is maximally induced in long-term cultures of theca interna cells following 72 h treatment with forskolin or cyclic AMP analogs (McAllister *et al.*, 1989, McAllister and Simpson., 1993). 17α-hydroxyprogesterone was used in these studies as an indirect reflection of 17α-hydroxylase activity. In normal long-term cultures we have previously published the close correlation of 17α-hydroxylase activity with 17α-hydroxyprogesterone production. Hence, in these experiments, 17α-hydroxyprogesterone values were measured under optimal conditions, 72 h following treatment. Forskolin, an activator of adenylate cyclase was used in these studies as a means of increasing intracellular cyclic AMP.

Statistical analysis was performed using ANOVA followed by identification of specific differences by Bonferonni's multiple *t*-test (one-sided).

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