Oestradiol synthesis by granulosa cells from immature rats treated with pregnant mare's serum gonadotrophin

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Abstract. Granulosa cells harvested from follicles in hypophysectomized or intact immature rats treated with 20 IU of pregnant mare's serum gonadotrophin (PMS) produced immunoreactive oestradiol (E₂) when incubated in Krebs Ringer bicarbonate buffer containing an NADPH generating system; inclusion of steroid substrates in the medium increased the rate of synthesis. Further, tritiated E₂ was synthesized when labelled progesterone was used as substrate. Granulosa cells removed from pre-ovulatory follicles on the morning of pro-oestrus in adult females also produced E₂ in vitro. Although E₂ synthesis was apparent by cells from immature hypophysectomized rats within 12 h of PMS treatment, it increased greatly with longer in vivo exposure to the gonadotrophin. Production was linear with the number of cells incubated and with time, at least through the first 30 min; the production rate decreased slightly with longer incubations. Exposure of the cells in vivo to hCG or ovine LH, before incubation, destroyed most of their ability to synthesize E₂ even if progesterone or pregnenolone was added to the medium, but conversion of testosterone to E₂ was reduced by only about 50%. Inhibitors of steroid synthesis, i.e. 4-OH-androstenedione, SU-10603, cyanoketone, or aminogluthethimide, greatly reduced the amount of E₂ synthesized by the cells. The results indicate that granulosa cells exposed in vivo to gonadotrophins can synthesize E₂ without the addition of androgenic substrate provided that cofactors are supplied. This finding has important implications for the current 'two cell' theory for oestrogen production by the ovary. A deficiency in steroidogenic enzymes within the granulosa cell appears to be an inadequate basis for the theory. However, the total synthesis of E₂ in vivo by granulosa cells has not been shown.

The original in vivo studies of Falck (1959), using tissue transplants and a biological response, suggested that two kinds of cells were involved in ovarian oestrogen production in the rat. Subsequent in vitro studies (extensively reviewed by Armstrong & Dorrington (1977), Dorrington & Armstrong (1979) and Richards (1979)) have attempted to explain the interaction of the cells on the basis of enzyme differences. According to the current 'two-cell' theory for the rat ovary, granulosa cells are deficient in two key enzymes, 17α-hydroxylase and C17-20 lyase, for conversion of progestins (pregnenolone and progesterone) to androgens. On the other hand thecal-interstitial cells lack aromatase (oestrogen synthesizing enzymes) for the conversion of the androgens they produce (androstenedione and testosterone) into oestrone (E₁) and oestradiol (E₂). However, when the cells are combined the androgen from the thecal-interstitial cells becomes the substrate for the aromatase activity of the granulosa cells. This attractive concept has been extensively used to interpret results obtained with cell cultures (Makris & Ryan 1975; Erickson & Hsueh 1978; Dorrington & Armstrong 1979; Richards 1979; Bogovich & Richards 1982).

Opposing this theory, recent studies (Johnson et al. 1981) have demonstrated that rat granulosa cells have 17α-hydroxylase activity. Furthermore, on
the basis of the amount of substrate converted under saturating conditions their 17α-hydroxylase and aromatase activities were about equal. Combining these data with the evidence which is accumulating that, at least in the testis, 17α-hydroxylase and C17-20-lyase activity reside in the same enzyme (Nakajin & Hall 1981; Chasalow et al. 1982) provides the suggestion that granulosa cells may have the complete steroidogenic enzyme system for oestrogen production. The present study was undertaken to demonstrate in vitro oestrogen synthesis by granulosa cells which have been exposed in vivo to gonadotrophic stimulation. The results clearly indicate that granulosa cells can synthesize E2 from progestins.

**Materials and Methods**

Immature (27 days) rats of the Holtzman strain obtained from Sasco Inc. (Omaha, NE) were injected (sc) with 20 IU of pregnant mare's serum gonadotrophin (PMS) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 ml of 0.15 M saline. In some experiments the animals were hypophysectomized, via the parapharyngeal approach, the day before injecting the PMS. At various times after administration of the PMS animals were decapitated and their ovaries placed as quickly as possible in ice-cold Krebs-Ringer bicarbonate buffer (KRB) (pH 7.4) containing 1 mg/ml of glucose and phenol red as a pH indicator. Cells from adult cycling females were also used. The animals were followed for three cycles before they were sacrificed on the morning of pro-oestrus.

Following removal of the oviduct and surrounding fat from the ovary, the large pre-ovulatory follicles were punctured with a sharpened No. 1 insect needle and the granulosa cells expressed into KRB using gentle pressure applied with a watch-maker's forceps. The cells from the ovaries of 15–30 rats were pooled in a 16 x 100 mm glass tube and collected by centrifugation at 200 x g for 5 min. The cells were washed twice, suspended in the same buffer and aliquot quantities pipetted into tubes for assay. The protein content of the suspension was determined, following rupture of the cells by sonication, using the Bradford (1976) method, with bovine serum albumin as the standard.

Glass culture tubes (12 x 75 mm) were used for incubating the cells. Steroid substrates, when used, were dissolved in methanol containing a small amount of Tween-80 and taken to dryness in the tubes prior to the addition of cells. The solvent alone was used in control tubes. The incubation fluid (100 µl) contained, in addition to the cells an NADPH generating system required for steroidogenesis (Gower 1975); i.e. NADP (1 mM), glucose-6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (0.1 U). The tubes were gassed with 95% O2 + 5% CO2, capped with paraffilm, and incubated at 37°C in a Dubnoff shaking water bath. The incubation was terminated by addition of 2 ml of reagent grade diethyl ether: [3H]oestriol was added at this time for calculation of extraction efficiency which averaged 85.5 ± 1.2% with a range of 69.9 to 95.4%. Controls includes tubes without cells as well as tubes containing cells, not incubated but extracted to determine the starting level of E2. Preliminary experiments determined that the amount of E2 in the culture media was proportional to the amount of cells incubated.

After complete mixing of the cells with ether the aqueous phase was frozen in an acetone + dry ice bath. The ether phase was decanted into a 12 x 75 mm tube and taken to dryness in a Vortex-evaporator (Buchler Instruments, Fort Lee, NJ) and the residue re-dissolved in 2 ml of methanol. Aliquot portions of this solution were assayed for steroid content.

Added evidence for the production of E2 was obtained by incubating cells with [1,2,6,7-3H]progesterone (97.9 Ci/mmol; New England Nuclear Corp. Boston, MA). The medium was extracted with ether and subjected to chromatography on 0.6 x 30 cm columns of Sephadex LH-20 using benzene-methanol (95:5, v/v) for elution. The fraction containing E2 was taken to dryness and re-dissolved in methanol. One portion was used for assay of E2 content and one portion was used for determination of radioactivity.

For radioimmunoassay [2,4,6,7-3H]Nα-oestradiol (115 Ci/mmol) and [2,4,6,7-3H]Nα-oestrone (104 Ci/mmol) were purchased from New England Nuclear Corp. and used without further purification. Unlabelled steroids were purchased from Sigma, as were NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Purity of steroids was checked by melting point determinations as well as by chromatography on silica gel.

Steroids were measured by radioimmunoassay. The characteristics of the antibodies used and the details of the procedure have been reported (Sashida & Johnson 1976). The minimal amount of E2 detectable was 2.5 pg; the intra-assay coefficient of variation did not exceed 5% and the inter-assay variation did not exceed 10%. The amount of steroid in extracts of incubation media plus cells was expressed as ng per mg protein. All of the extracts from one experiment were assayed simultaneously. Differences between group means in the same assay with a P value of less than 0.05, using Student's t-test were considered statistically significant.

**Results**

Early trials indicated the necessity for the addition of cofactors to obtain E2 synthesis in vitro. Cells
The effect of adding cofactor on E₂ production by granulosa cells. A pool of cells was obtained from the follicles of intact immature rats 48 h after injection of 20 IU of PMS. After washing, the cells were suspended in KRB buffer (pH 7.4) containing glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Initial concentration of E₂ is indicated on the left (o). Addition of NADP greatly increased E₂ production. Neither NAD nor nicotinamide (NA) could replace the NADP. Addition of NAD to NADP did not increase production. SEM for groups of 4 cultures are indicated by vertical lines.

Granulosa cells removed from the follicles of hypophysectomized rats 12 h after the administration of PMS containing 0.64 ± 0.04 ng E₂ per mg of protein. Incubation of these cells for 60 min without the addition of exogenous substrate yielded 1.43 ± 0.24 ng/mg, or a production of 790 pg of E₂ per mg of protein. Inclusion of 300 ng of progesterone in the medium nearly quadrupled the amount of E₂ produced (3.04 ± 0.15 ng/mg). Inclusion of testosterone (1000 ng) increased E₂ production significantly to 8.36 ± 0.9 ng/mg (P < 0.05 compared with progesterone). With all studies involving added substrate, control tubes containing the steroid were incubated, without the addition of cells, extracted and assayed for E₂ content; E₂ was undetectable in these tubes.

The pooled cells from another series of 20 rats were harvested and incubated 24 h after injection of PMS. Cell E₂ content was 2.01 ± 0.14 ng/mg. Incubation of these cells without substrate pro-

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**Fig. 1.**

**Fig. 2.**

Oestradiol (E₂) content in and production by pooled granulosa cells from groups of 20 immature hypophysectomized rats removed 24, 48 or 72 h after injection of 20 IU of PMS and incubated for 60 min. Open bars ("o") indicate the amount of E₂, as ng/mg protein, in non-incubated cells. The incubation medium (KRB) was used with or without pregnenolone. Note the different scales for each time period.

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duced 2.5 ng/mg but with 1000 ng of pregnenolone E₂ production nearly doubled again (Fig. 2, 24 h). The E₂ content of cells from another group of 25 animals injected with PMS at the same time as those above, but removed at 48 h, was 3.85 ± 0.29 ng/mg protein (Fig. 2, 48 h). Incubation of aliquot portions of these cells for 60 min without added substrate resulted in production of 18.9 ± 1.44 ng/mg; the amount of E₂ produced increased to 32.14 ± 4.5 ng/mg with pregnenolone (1000 ng) in the medium (Fig. 2). If the granulosa cells were obtained 72 h after treatment with PMS (another 20 rats from the same series of animals as used above) the E₂ content was increased to 20.8 ± 1.6 ng/mg (Fig. 2, 72 h). Incubation of these cells nearly quadrupled the E₂ content of the media and 56.2 ± 4.4 ng/mg was produced during a 60 min incubation. Inclusion of pregnenolone (1000 ng) increased production to 99.9 ± 7.8 ng/mg protein.

The pooled cells of another group of 25 hypophysectomized rats, removed 48 h after PMS, contained 2.44 ± 0.11 ng/mg of E₂. Incubation of these cells for 60 min with 100 ng of progesterone

Oestradiol (E₂) and oestrone (E₁) synthesis, as a function of time, by granulosa cells obtained from hypophysectomized immature rats 48 h after injection of 20 IU of PMS. The incubation medium contained 900 ng of progesterone as substrate. The amount of E₂ and E₁ at time 0 was substracted from the amount present at the termination of each incubation.

Oestradiol (E₂) production by pooled granulosa cells removed from intact immature rats either 48 or 60 h after injection of PMS and incubated for 60 min with or without progesterone (Prog.), pregnenolone (Preg.) or testosterone (Test.) as substrate. Animals supplying cells at 60 h had received (ip) 10 µg of ovine LH at 48 h. The amount of E₂ (ng/mg protein) in non-incubated cells is shown on the left ("O").
yielded 61.8 ± 3.9 ng/mg, or a production of 59.4 ng/mg of E2. These cells were incubated with 900 ng of progesterone for varying periods (Fig. 3). Production was linear for the first 30 min and then began to decline. E1 production, which was less than 10% that of E2, was linear between 30 and 90 min of incubation. This experiment was repeated with similar results.

Some of the animals, injected with PMS at the same time as those used in the above timing experiment, were injected (sc) with 10 IU of hCG 48 h after PMS and their cells harvested 24 h later. The E2 content of the cells fell by 70% to 0.72 ± 0.03 ng/mg of protein, compared to that obtained before injection of hCG. Incubation with 100 ng of progesterone produced an E2 level of 3.71 ± 0.13 ng/mg or a production of 2.99 ± 0.10 ng/mg in 60 min. This was a 95% reduction from the production seen with cells removed prior to hCG treatment.

The effect of exposure to a luteinizing agent on granulosa cell oestrogen production was examined further using intact females. Cells were harvested 48 h after PMS and also at 60 h, 12 h after injection (ip) of 10 μg of ovine LH (NIH-S-18). Fig. 4 shows the decrease in cellular content and the loss of E2 production efficiency after exposure of the cells to LH. While production fell by nearly 90% when no substrate was provided, or when progesterone or pregnenolone were included, there was only a 52% reduction with testosterone as substrate.

In order to help establish that E2 was being synthesized from exogenous sources, cells were incubated with labelled progesterone. The pooled granulosa cells from 10 hypophysectomized rats were obtained 72 h after injection of PMS. Ten aliquot portions of the cell suspension, each containing 168 μg of cellular protein, were incubated along with 1.04 ng of tritiated (7.13 × 10⁵ DPM) and 10 ng of unlabelled progesterone. After incubation for 60 min the cells and media were extracted with ether and the E2 separated by chromatography. When assayed by RIA the E2 fraction contained 14.74 ± 0.44 ng which translates into a production rate of 87.5 ± 2.6 ng/mg protein. The amount of [3H]E2 in the fraction was 6.171 ± 0.154 × 10⁴ DPM, or 8.6% of the total label added.

If the cells synthesize E2 from progesterone then inhibitors should block this production: four agents were used to test this. 4-OH-androstenedione was used to inhibit aromatase, cyanoketone to inhibit 3β-ol-hydroxysteroid dehydrogenase + Δ⁴⁻⁵ iso-}

**Fig. 5.** Oestradiol synthesis, as a per cent of control, in cultures containing 300 ng of progesterone as substrate and in addition either 4-OH-androstenedione to inhibit aromatase, cyanoketone to inhibit 3-OH-steroid dehydrogenase-isomerase, SU-10603 to inhibit 17-hydroxylase and/or C17-20-lyase, or aminoglutethimide to inhibit all cytochrome P-450 dependent enzymes.
The one demonstrated substrates gens nenetone, produce progesterone one. The produced PMS 1977; 1976; significant using explanation strates present. In injection h. increased production (their cultured Hambger & Armstrong 1977; Nimrod 1977; Hamberger et al. 1978). Because progestin substrates were supplied in most cultures in the present study, attention was not directed toward progesterone production. However, the cells did produce this steroid. For example, the cells removed from follicles of intact females 48 h after PMS (their E₂ production is shown in Fig. 4) produced 136.2 ± 7.8 ng progesterone mg protein per h. At 60 h, after the endogenous LH surge and the injection of 10 μg of ovine LH, the production rate increased to 322.2 ± 20.2 ng/mg progesterone.

In contrast to progesterone, most (Erickson & Hsueh 1978; Dorrington & Armstrong 1979; Hillier et al. 1980), but not all (Makris & Ryan 1975; Hamberger et al. 1978; Carson et al. 1981), studies using cultured granulosa cells have failed to obtain significant E₂ synthesis unless androgenic substrates were provided. Therefore we must seek an explanation for the E₂ produced by the cells in the present study. First, E₂ production may be a consequence of impure granulosa cell cultures. That is, contaminating thecal-interstitial cells could supply, or produce, androgens which could be converted to oestrogens by the aromatase in the granulosa cells. Such contamination is difficult to rule out or to assess, but granulosa cells from the large follicles are rather easily obtained with only a small puncture wound making heavy contamination unlikely.

One might argue that the granulosa cells obtained androgens from the thecal-interstitial cells before incubation and that they merely converted these substrates into E₂. Such a source of substrate needs consideration. Fig. 2 shows the result of a typical incubation with production of about 20 ng of E₂ per mg of cellular protein. These cells contained 2.21 ± 0.16 ng of immunoreactive testosterone and 3.42 ± 0.08 ng of androstenedione per mg of protein when they were removed from the ovaries. If we assumed that this androgen was of thecal-interstitial cell origin, rather than synthesized in the cells, it would account for less than half the E₂ found at the end of the incubation.

Another important difference in the present study is the inclusion of cofactors in the incubation medium. The entrance of cofactor into the cells was tested by use of an NADPH generating system together with nitroblue tetrazolium (Aldred & Cooke 1982); > 75% of the cells gave a positive

**Table 1.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cellular oestradiol content</th>
<th>Cells plus incubation medium</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Without substrate</td>
<td>With substrate</td>
</tr>
<tr>
<td>Progesterone 1</td>
<td>1.52 ± 0.1*</td>
<td>2.56 ± 0.19</td>
</tr>
<tr>
<td>Pregnenolone 2</td>
<td>1.60 ± 0.1</td>
<td>4.75 ± 0.11</td>
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* Results expressed as ng/mg protein per h ± SEM for groups of 4 cultures.

** Indicates a P < 0.005 compared to no substrate groups. Groups of 6 rats were followed for 3 consecutive cycles and killed on the morning of pro-oestrus. Granulosa cells were removed from the largest follicles and cultured in KRB buffer with cofactors and with or without added substrate. E₂ was extracted and assayed by RIA.
diaphorase response within 30 min. While this indicates that the cell membranes were 'leaky' for these large molecules it does not distract from our consideration of the cellular content of enzymes necessary for E₂ synthesis.

Why should the androgen synthesizing enzymes in the cells require exogenous NADPH while their aromatase apparently does not? There are abundant reports of granulosa cell cultures which included testosterone or androstenedione, but not cofactor, for the successful production of E₂. We do not have an adequate answer for this but it may only involve a quantitative difference in cofactor requirements. In our previous studies, measuring enzyme activities in granulosa cells, we found that about 15% of their aromatase but none of their 17α-hydroxylase activity was expressed without added NADP (Johnson et al. 1981).

While the amount of E₂ produced was proportional to the number of cells incubated, the amount produced was linear with time of incubation only during the first 30 min. The reason for the gradual decline in rate of production at longer incubation times is unknown, but a loss of E₂ to E₁ did not account for the change with time (Fig. 3). In an early experiment, in which the number of cells incubated and the amount of E₂ produced was less than that shown in Fig. 3, the production rate was linear through 90 min (data not shown); these data provided the reasoning for using 60 min for many of our subsequent incubations.

Previous studies (Sashida & Johnson 1976; Johnson & Cheng 1978) have shown that a 12–16 h lag period is necessary for production of oestrogen by the immature rat ovary after the injection of PMS. The present study has shown that exposure of the granulosa cells for 12 h to this gonadotrophin, which has both FSH and LH actions in this species, is sufficient to induce all of the enzymes necessary for E₂ synthesis. The synthetic capacity increased rapidly with time of in vivo exposure to PMS with the cells removed at 72 h having a 15-fold increase in production compared to those removed at 24 h (Fig. 2).

Inhibitors of steroid synthesis were used in order to give added evidence that the cellular enzymes were actually synthesizing the oestrogen. Amino-glutethimide, an inhibitor of cytochrome P450 (Thompson & Siiteri 1974), very effectively removed most of the E₂ production. SU-10603, which inhibits 17α-hydroxylase/C17,20-lyase activity (Gower 1975), also removed E₂ synthesis by the cell incubates. Cyanoketone, an inhibitor of 3β-ol-hydroxysteroid dehydrogenase + Δ⁴,5-isomerase (Goldman et al. 1973) reduced E₂ production by about 50% even though progesterone had been included in the incubation medium. The dose of cyanoketone used inhibits microsomal C17,20-lyase activity of ovarian homogenates by nearly 60% (Johnson & Griswold, unpublished data) which would explain the results. However, the mechanism of the inhibition remains to be determined.

Another test of the oestrogen synthesizing system employed the use of LH prior to removal of the cells from the ovary. The ovulatory surge of LH on pro-oestrus reduces ovarian oestrogen production presumably because of loss of 17α-hydroxylase and/or C17-20-lyase activities (discussed by Ahrén et al. 1979). We have shown (Johnson 1978; Tsai-Morris & Johnson 1982) that the ovulatory surge in LH or treatment with LH causes a rapid and dramatic loss in 17α-hydroxylase activity: this loss would obviously remove the ability of the cells to produce E₂. After in vivo exposure to hCG or LH the cells synthesized much less E₂, but their ability to aromatize testosterone was only reduced. Therefore, the reduction in oestrogen synthesis associated with ovulation is more likely a result of a reduced 17α-hydroxylase and/or C17-20-lyase activities than to the reduction in aromatase activity (Hamberger et al. 1978; Suzuki & Tamaoki 1980).

The doses of progestins used in the present study were varied. In our previous studies (Johnson et al. 1981) we found that with 17α-hydroxylase and aromatase about 1 nmol of substrate was converted per mg of cellular protein per h. In an attempt to assure that the cells had sufficient substrate we used between about 1 to 3 nmols of substrate; we did not check the solubility of the steroids but assumed that the incubation medium was saturated.

The demonstration that granulosa cells can synthesize oestrogen in vitro when the necessary cofactors are provided does not prove that these cells have such a function in vivo. On the other hand, failure to demonstrate oestrogen production in vitro without providing essential cofactors cannot be used to prove that the cells do not synthesize oestrogens in vivo. The important point is that the 'two-cell theory' as currently formulated (Dorrington & Armstrong 1979; Bogovich & Richards 1982) is only one possible explanation for oestrogen production. Certainly the basis for the theory in enzyme deficiencies needs further consideration in
view of the present results combined with those previously obtained (Johnson et al. 1981). While thecal-interstitial cells may augment the action of granulosa cells for the production of oestrogens (Makris & Ryan 1975) the mechanisms involved have not been resolved. The cells used in the present study were differentiated in the presence of thecal-interstitial tissues. Cellular cooperation not involving steroid exchanges may be involved and require further experimentation.

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References


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