Effects of Androgen on 17β -Estradiol Production by Cultured Human Granulosa Cells

YUICHI WADA, AKIRA TSUIKI, TAKAO FUKAYA, OSAMU
SHINKAWA, SHINJI SATOH, MASAYUKI HORIGUCHI, HIROSHI
HOSHIAI and AKIRA YAJIMA

*Department of Obstetrics and Gynecology, Tohoku
University School of Medicine, Sendai 980*

WADA, Y., TSUIKI, A., FUKAYA, T., SHINKAWA, O., SATOH, S., HORIGUCHI, M.,
HOSHIAI, H. and YAJIMA, A. *Effects of Androgen on 17β -Estradiol Production by
Cultured Human Granulosa Cells.* Tohoku J. exp. Med., 1988, **154** (3), 253-260

— In order to determine whether androgen acts solely as a substrate for aromatization or whether it also influences on the activity of aromatase enzyme, human granulosa cells were incubated in vitro with or without androgen. Although basal production of 17β -estradiol (E_2) in cultured granulosa cells obtained from follicles about 26 hr after the initiation of the LH surge was restricted in small quantities, a marked increase in E_2 production occurred in the presence of testosterone (T) (10^{-6} M) as aromatizable substrate. The non-aromatizable androgen, 5α -dihydrotestosterone (DHT) (10^{-7} M, 10^{-6} M), slightly enhanced E_2 production and it did not inhibit T aromatization in these cells. By contrast, DHT did not increase E_2 production in granulosa cells obtained from follicles 2-5 days before the LH surge. The results indicate that androgen can enhance the estrogen biosynthesis of granulosa cells obtained from follicles during the LH surge not only by acting as a substrate for aromatization but also by participating in some process of the estrogen synthesis. This effect of androgen was not clearly seen in granulosa cells obtained before the LH surge, suggesting that the response of granulosa cells to exogenous androgen varies with the stage of the cell differentiation. —————
granulosa cells; dihydrotestosterone; testosterone

Androgen, together with gonadotropins and estrogen, controls the development and maturation of ovarian follicles. In general, under increased levels of androgen, which inhibits the effect of estrogen, ovarian follicles become atretic (Louvét et al. 1975), and show a high androgen/estrogen ratio in their microenvironment (Brailly et al. 1981). Testosterone (T) aromatization in human cultured granulosa cells is suppressed by the addition of the non-aromatizable androgen dihydrotestosterone (DHT) (Hillier et al. 1980a, b). Thus, a suppressive effect of androgen on follicular development has occasionally been reported. On the other hand, physiologically some androgens (aromatizable androgens) work as substrates in granulosa cell aromatization. Further, it has been reported that androgen can

Received December 5, 1987; revision accepted for publication February 15, 1988.

augment the stimulatory effect of FSH on the aromatase system in cultured rat granulosa cells (Daniel and Armstrong 1980). Thus, the effects of androgen on the follicular estrogen biosynthesis seem to be controvertible.

In the present study, we cultured granulosa cells obtained from the ovarian follicles of women in the two different stages around ovulation and compared the effects of androgen on 17β -estradiol (E_2) production.

MATERIALS AND METHODS

Subjects

The subjects were 25 infertile women without any underlying endocrine abnormalities comprising 18 who underwent IVF & ET and 7 who underwent laparoscopy in our department. Granulosa cells were obtained from them according to the following procedure.

Method for sampling of granulosa cells

In the 18 women who underwent IVF & ET, follicular development was promoted by administration of clomiphene citrate at a dose of 100 mg for 5 days from the fifth day of the menstrual cycle. The diameter of the ovarian follicle was measured every day by ultrasonography. After maturation of the follicle had been confirmed, the onset of the midcycle LH surge was judged by semi-quantification of urinary LH by means of a Hi-gonavis kit (Mochida Seiyaku Co., Ltd., Tokyo). About 26 hr after the initiation of the LH surge, follicular puncture was carried out under laparoscopic guidance. The follicular fluid was aspirated and the ovum was sampled. Granulosa cells were then collected from the remaining follicular fluid and washings of the follicular antrum.

Furthermore, 7 patients in the mid-follicular phase (2–5 days before the last day of low BBT) who had not been treated with clomiphene citrate during the menstrual period, were also examined. The follicular fluid was aspirated by puncture from several follicles under laparoscopic guidance, and granulosa cells were collected. Cell culture was carried out for only 4 of 7 patients, since the number of cells was insufficient in the other three.

Method of cell culture

An outline of the method used for granulosa cell culture has been reported previously (Wada et al. 1986). Briefly, the follicular fluid and washings of the follicular antrum were centrifuged ($150 \times g$, 6 min), and the supernatant was removed using sterile techniques. To the sediment, about 2 ml of ACK buffer solution (0.15 M NH_4Cl , 10 mM $KHCO_3$, 0.13 mM EDTA) was added to eliminate red blood cells. After centrifugation, the sediment was washed with about 2 ml of 200 $\mu g/ml$ penicillin-supplemented Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, NY, USA). The washing procedure was performed three times, and cells were resuspended by syringing for each washing. Some of the resuspended cells were stained with 0.5% trypan blue in order to calculate the cell number and the rate of cellular viability. The rate of cellular viability was about 40–50%. Cell culture was begun with 1 ml of a cell suspension of $0.5\text{--}2 \times 10^5$ viable cells/ml in the Nunclon tissue culture dish measuring 35 mm in inside diameter in a CO_2 incubator with a 5% $CO_2/95\%$ air atmosphere at 37°C. The culture medium was the above-mentioned washing medium supplemented with 20% fetal calf serum (Gibco). The entire culture medium was renewed every 24 or 48 hr and then kept at $-20^\circ C$ until steroid determination.

Addition of hormones

In order to examine the effects of the addition of hormones, 10^{-6} M testosterone (T ;

Sigma Chemical Co., St. Louis, MO, USA) and 10^{-7} M or 10^{-6} M 5 α -dihydrotestosterone (DHT; Sigma) were added in vitro. In each experiment, E₂ production was compared between cells from the same culture to which hormones were or were not added.

Determination of steroid and statistics

E₂ in the culture medium was determined by means of a 17 β -estradiol RIA kit (CIS, Italy) using a double-antibody method with ¹²⁵I as a tracer (Uehara et al. 1985). For the values obtained, the mean $\pm$ s.e. was calculated, and statistical analysis was carried out by paired *t*-test, regarding $p < 0.05$ as the level of statistical significance.

RESULTS

Production of basal E₂ by cultured granulosa cells

Granulosa cells obtained from ovarian follicles under the LH surge produced E₂ at a rate of 20.0 ± 3.9 fg/cell during the first 2 days of culture, but the production decreased linearly thereafter. In comparison with this, E₂ production by granulosa cells before ovulation was slightly low, 7.1 ± 2.4 fg/cell, during the initial 2 days, but there was no great difference thereafter (Fig. 1).

Effects of androgen on E₂ production by granulosa cells obtained from preovulatory follicles during the LH surge

In order to examine the effects of androgen on E₂ production by cultured granulosa cells obtained from follicles during the LH surge, DHT, a non-aromatizable androgen, was added to the culture medium, and changes in E₂

Fig. 1. The amounts of E₂ produced over a 6-day period in vitro by human granulosa cells

The production by granulosa cells obtained from preovulatory follicles during the midcycle LH surge ($\square$, mean $\pm$ s.e. of 10 cases).

The production by granulosa cells obtained from follicles 2-5 days before the LH surge (hatched , mean $\pm$ s.e. of 4 cases).

Fig. 2. Effects of 10^{-6} M (a) or 10^{-7} M (b) DHT on E_2 production by human granulosa cells obtained from preovulatory follicles during the LH surge.

* $p < 0.05$; ** $p < 0.01$.

against E_2 production without DHT.

production were compared with those in the control containing no androgen. In the following androgen-supplementation experiments, the initial 2 days were used as a period of preliminary culture in order to lessen any *in vivo* influences, and the hormone was added to the culture medium from the third day. During the test period there was no particular difference in cell growth between two groups.

As shown in Fig. 2, E_2 production was slightly, but significantly, increased in the presence of 10^{-6} M and 10^{-7} M DHT from the second day as compared with the

Fig. 3. Effects of 10^{-6} M T alone or 10^{-6} M T plus 10^{-6} M DHT on E_2 accumulation by human granulosa cells obtained from preovulatory follicles during the LH surge (mean $\pm$ s.e. of 4 cases).

Fig. 4. Effects of 10^{-6} M DHT on E_2 production by human granulosa cells obtained from follicles 2-5 days before the LH surge (mean $\pm$ s.e. of 4 cases).

levels without the hormone. The E_2 production at 4 days of 10^{-6} M DHT addition was about five times greater than that in the absence of the hormone.

Androgen has been reported to suppress E_2 production by inhibiting the aromatizing enzyme activity of granulosa cells (Hillier et al. 1980a, b). In this regard, the E_2 production in the presence of T, an aromatizable androgen, was compared with E_2 accumulation in the presence of both T and DHT (Fig. 3). E_2 production was markedly increased in the presence of 10^{-6} M T alone as compared with that in the absence of T. E_2 production in the presence of both 10^{-6} M T and 10^{-6} M DHT was not suppressed but tended to be slightly promoted when compared with that in the presence of T alone. E_2 accumulation at 2 days was 3.1 ± 0.6 fg/cell in the absence of hormones, 138.1 ± 28.7 fg/cell in the presence of T alone, and 185.1 ± 9.1 fg/cell in the presence of T + DHT.

Effects of androgen on E_2 production by granulosa cells before the LH surge

There were no significant influences of DHT (10^{-6} M) on E_2 production by granulosa cells obtained from ovarian follicles before the LH surge (Fig. 4). However, marked suppression of E_2 production was found in 3 of 4 cases one day after DHT addition. In these 3 cases, granulosa cells were obtained 3-5 days before the last day of low BBT, whereas in the remaining case the corresponding cells were obtained 2 days before the last day of low BBT. In addition, in one of the three cases showing suppression, E_2 production was lower in the presence of DHT 2 and 3 days after DHT addition than in the absence of the hormone. In contrast, in the other 2 cases and the case in which E_2 production was not suppressed on the first day, E_2 production was rather increased 3 days after DHT addition.

DISCUSSION

At around the time of the midcycle LH surge, ovarian follicles show a tendency to luteinization, and the steroid environment shifts from an E_2 -dominant to a P-dominant situation. In the present study, granulosa cells obtained from ovarian follicles during the LH surge were shown to produce a large quantity of E_2 in the presence of T, the substrate of aromatization. Therefore, it was confirmed that granulosa cells possess sufficient aromatase activity while shifting to a P-dominant situation at the ovulatory period. Also, in the human hMG-hCG cycle, it has been confirmed that granulosa cells sampled just after hCG injection have high aromatase activity (Dlugi et al. 1984; Polan et al. 1984).

In the present study, we used the non-aromatizable androgen DHT in order to examine the direct effect of androgen on granulosa cells. E_2 production by granulosa cells obtained from follicles before the LH surge did not show any significant response to the addition of DHT alone at 10^{-6} M, but E_2 production 24 hr after DHT addition was lower than that without DHT in 3 of 4 cases. On the other hand, E_2 production by granulosa cells obtained from ovarian follicles during the LH surge showed a slight but significant increase in E_2 production in the presence of 10^{-6} – 10^{-7} DHT. Similarly a slight increase in T aromatization by addition of DHT was observed.

Hillier et al. (1980b) performed incubation experiments using granulosa cells obtained from follicles at 9 days (ϕ 5–9 mm diameter) and 13 days after the start of menstruation (ϕ 20 mm diameter) in order to examine the effects of DHT. It was found that the increased production of E_2 following the addition of T and FSH, an aromatase inducer, to the culture medium, was suppressed in cells from both stages in the presence of DHT (10^{-5} – 10^{-6}), indicating competitive inhibition of T aromatization by DHT.

It has been demonstrated that DHT exerts its action by binding to the androgen receptor of the ovary (Schreiber and Ross 1976). However, the possibility that DHT exerts its action by binding to an aromatase complex or enzymes such as 3α -hydroxysteroid dehydrogenase has been suggested (Jarrell and Belbeck 1985), and it is suspected that the reaction mode might be more complicated when the culture period is prolonged. The data of Hillier et al. (1980b) were obtained for 3 hr of incubation, whereas our present data were on the difference in production obtained every 24 hr. Therefore, it is not possible to directly compare their data with ours.

However, there was an evident difference in the reaction against DHT under the same culture conditions between granulosa cells before and during the LH surge. Therefore, there is a possibility that the reactivity of granulosa cells during the LH surge, which have high aromatase activity, differs from that of granulosa cells obtained from immature or atretic ovarian follicles. As a background factor, the effects of intrinsic gonadotropin must naturally be considered.

FSH induces aromatase in granulosa cells (Armstrong and Papkoff 1976 ; Moon et al. 1978 ; Hillier et al. 1980b). Also, granulosa cells morphologically differentiate from autosynthetic cells into steroid-secreting cells around the time of the LH surge (Wada et al. 1986), and the amount of gonadotropin receptors increases markedly along with this change (Richards and Midgley 1976). Variations in the reaction of granulosa cells to steroid according to the maturity of the sampled cells have also been reported by Jarrell and Belbeck (1985) and Bieszczad et al. (1982).

The significance of androgen on the process of ovarian follicle maturation during the time of ovulation remain unclear. We have previously confirmed that with regard to cultured granulosa cells obtained at ovum sampling for IVF & ET, P production stimulated by hCG is suppressed by the simultaneous addition of T (Wada et al. 1988). Taking this into consideration together with the findings of the present study, it is speculated that androgen controls the early switch from an E₂-dominant to a P-dominant situation in ovarian follicles at the local level.

References

- 1) Armstrong, D.T. & Papkoff, H. (1976) Stimulation of aromatization of exogenous and endogenous androgen in ovaries of hypophysectomized rats *in vivo* by follicle-stimulating hormone. *Endocrinology*, **99**, 1144-1151.
- 2) Bieszczad, R.R., McClintock, J.S., Pepe, G.T. & Dimino, M.J. (1982) Progesterone secretion by granulosa cells from different sized follicles of human ovaries after short term incubation. *J. clin. Endocr.*, **55**, 181-184.
- 3) Brailly, S., Gougeon, A., Milgrom, E., Bomsel-Helmreich, O. & Papiernik, E. (1981) Androgens and progestins in the human ovarian follicle : Differences in the evolution of preovulatory, healthy nonovulatory and atretic follicles. *J. clin. Endocr.*, **53**, 128-134.
- 4) Daniel, S.A.J. & Armstrong, D.T. (1980) Enhancement of follicle-stimulating hormone-induced aromatase activity by androgens in cultured rat granulosa cells. *Endocrinology*, **107**, 1027-1033.
- 5) Dlugi, A.M., Laufer, N., Polan, M.L., DeCherney, A.H., Tarlatzis, B.C., MacLusky, N.J. & Berhman, H.R. (1984) 17 β -estradiol and progesterone production by human granulosa-luteal cells isolated from human menopausal gonadotropin-stimulated cycles for *in vitro* fertilization. *J. clin. Endocr.*, **59**, 986-992.
- 6) Hillier, S.G., van den Boogaard, A.M.J., Reichert, L.E., Jr. & van Hall, E.V. (1980a) Alterations in granulosa cell aromatase activity accompanying preovulatory follicular development in the rat ovary with evidence that 5 α -reduced C₁₉ steroids inhibit the aromatase reaction *in vitro*. *J. Endocr.*, **84**, 409-419.
- 7) Hillier, S.G., van den Boogaard, A.M.J., Reichert, L.E., Jr. & van Hall, E.V. (1980b) Intraovarian sex steroid hormone interactions and the regulation of follicular maturation : Aromatization of androgens by human granulosa cells *in vitro*. *J. clin. Endocr.*, **50**, 640-647.
- 8) Jarrell, J. & Belbeck, L. (1985) Evidence for a granulosa cell site of dihydrotestosterone metabolism in the adult rat ovary. *Biol. Reprod.*, **33**, 1207-1212.
- 9) Louvet, J.-P., Harman, S.M., Shreiber, J.R. & Ross, G.T. (1975) Evidence for a role of androgens in follicular maturation. *Endocrinology*, **97**, 366-372.
- 10) Moon, Y.S., Tsang, B.K., Simpson, C. & Armstrong, D.T. (1978) 17 β -estradiol biosynthesis in cultured granulosa and theca cells of human ovarian follicles : Stimulation by follicle-stimulating hormone. *J. clin. Endocr.*, **47**, 263-267.

- 11) Polan, M.L., Laufer, N., Ohkawa, R., Botero-Ruiz, W., Hasheltine, F.P., Decherney, A.H. & Behrman, H.R. (1984) The association between granulosa cell aromatase activity and oocyte-corona-cumulus-complex maturity from individual human follicles. *Endocrinology*, **59**, 170-174.
 - 12) Richards, J.S. & Midgley, A.R., Jr. (1976) Protein hormone action: A key to understanding ovarian follicular and luteal cell development. *Biol. Reprod.*, **14**, 82-94.
 - 13) Schreiber, J.R. & Ross, G.T. (1976) Further characterization of a rat ovarian testosterone receptor with evidence for nuclear translocation. *Endocrinology*, **99**, 590-596.
 - 14) Uehara, S., Naganuma, T., Chida, S., Yohkaichiya, T., Tsuiki, A., Kyohno, K., Mori, R., Imaizumi, H., Hoshiai, H. & Suzuki, M. (1985) The evaluation of serum estradiol, progesterone, testosterone concentrations and urinary estrogen excretion level for the monitoring indices of follicular maturation. *Acta obstet. gynec. jap.*, **37**, 1849-1858. (in Japanese with English abstract)
 - 15) Wada, Y., Tsuiki, A., Uehara, S., Oikawa, N., Hoshiai, H. & Yajima, A. (1986) Functional differentiation of granulosa cells of ovulatory follicles. *Clin. Endocr.*, **34**, 1113-1118. (Japanese)
 - 16) Wada, Y., Tsuiki, A., Uehara, S., Fukaya, T., Ishikawa, M., Hoshiai, H. & Yajima, A. (1988) Effect of androgen on 17β -estradiol and progesterone secretion by cultured human granulosa cells. *Acta obstet. gynec. jap.*, **40**, 331-337. (in Japanese with English abstract)
-