Effect of Adrenal and Ovarian Androgens on Type 4 Follicles Unresponsive to FSH in Immature Mice

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The present study investigates the physiological significance of dehydroepiandrosterone, dehydroepiandrosterone sulfate, T, androstenedione (Δ4), dihydrotestosterone (DHT), estrone (E1), and E2 on recombinant human FSH- (rhFSH) resistant type 4 follicles obtained from immature mice. Type 4 follicles of a diameter of 100–120 μm with one or two granulosa cell layers around the oocyte and an intact basal lamina with theca cells were isolated from the ovaries of 11-d-old BDF-1 mice and cultured with medium alone (control) or with dehydroepiandrosterone, dehydroepiandrosterone sulfate, T, Δ4, DHT, E1, or E2 at concentrations ranging from 1 × 10^{-11} to 1 × 10^{-7} M for 4 d. We examined the mean diameters of type 4 follicles, levels of immunoreactive (IR)-inhibin, and E2 and progesterone in the culture media on day 4. In addition, we evaluated follicular cell proliferation by immunofluorescence staining with 5-bromo-2'-deoxyuridine. All tested androgens significantly increased the diameter of type 4 follicles in a dose-dependent manner without the production of IR-inhibin and E2. The nuclei of granulosa cells in type 4 follicles cultured with all tested androgens exhibited intense 5-bromo-2'-deoxyuridine-positive staining, compared with those of controls. In contrast, neither E1 nor E2 had any stimulatory effects. The stimulatory effects of T, Δ4, or DHT were inhibited by an AR antagonist in a dose-related fashion but not by an aromatase inhibitor. Furthermore, all tested androgens had a synergistic effect on follicular growth and the production of IR-inhibin and E2. These results demonstrated that neither adrenal nor ovarian androgens are arteriogenic but that they stimulate type 4 follicles unresponsive to rhFSH and augment the responsiveness of these follicles to rhFSH. (Endocrinology 142: 4930–4936, 2001)

Because a single injection of T reduces ovarian weight and increases the number of atretic follicles in hypophysectomized E-treated immature rats (1, 2) and an injection of the nonaromatizable androgen, 5α-dihydrotestosterone (DHT) to normal cycling mice reduces the number of normal large follicles by 50% (3), ovarian androgens have long been implicated as an inhibitor of follicular development (4–6). Recent in vitro studies, however, have shown that T and DHT stimulate the production of progesterone by granulosa cells from hypophysectomized E-treated immature rats (7–9) and that ovarian androgens enhance the cyclic AMP production (10) as well as the aromatase activity (11, 12) of granulosa cells from normal and E-primed immature rats. In addition, ovarian androgens were found to stimulate the growth of small preantral mouse follicles cultured in vitro (13, 14) and T and DHT to increase the number of follicles as well as theca and granulosa cell proliferation in normal cycling rhesus monkeys (15). These results suggest that ovarian androgens have a stage-specific action on follicular development and can elicit a stimulatory action on follicular growth at the preantral stage. However, whether adrenal androgens have such effects on preantral follicular growth remains unknown. The fact that congenital adrenal hyperplasia causes ovarian dysfunction and premature adrenarche promotes the onset of puberty in women raises the notion that adrenal androgens are involved in ovarian function (16).

Very early follicular growth is gonadotropin independent (17), and the involvement of intra- and extraovarian factors such as activin or GH has been thought important in regulating the first step of folliculogenesis. In immature mice, intra- and extraovarian factors may stimulate small preantral follicles at a level at which they acquire responsiveness to FSH (18, 19). Studies of androgen-resistant testicular feminization mouse (Tfm) female mice have shown impaired reproductive performance, such as premature luteinization and progressive decrease in primordial follicles followed by premature cessation of reproduction (20, 21), although to the best of our knowledge, whether these animals have delayed puberty or a normal response to FSH remains to be clarified. Because ovaries have ARs at birth (22), understanding the role of androgens on follicular growth at very early stages is important for understanding the pathophysiology of prepubertal follicular growth. The present study investigates the physiological role of gonadal and adrenal androgens and estrone (E1) and E2 on follicular growth of immature mouse type 4 follicles that remain unresponsive to FSH.

Materials and Methods

Chemicals

We purchased 5-androsten-3β-ol-17-one (DHEA), 5-androsten-3β-ol-17-one sulfate (DHEAS), 4-androsten-17β-ol-3-one (T), 4-androsten-3,17-dione (Δ4), 5α-dihydrotestosterone (DHT), 1,3,5(10)-estratriene-3-ol-17-one (E1), and 1,3,5(10)-estratriene-3,17β-diol (E2) from Sigma (St.
Louis, MO). Recombinant human FSH (rhFSH) (lot no. 19503064) was supplied by Organon Co. (Oss, The Netherlands). The AR antagonist, OH-Flutamide, and the aromatase inhibitor, Fadrozole hydrochloride, were also supplied by Nippon Kayaku Co. (Tokyo, Japan) and Novartis Pharma Co. (Tokyo, Japan). All other chemicals were of analytical grade or of the highest quality commercially available.

**Animals**

BDF1 hybrid female lactating mice with 7-d-old female pups were purchased from Japan Charles River Laboratories, Inc. (Tokyo, Japan) and housed in a temperature- (24–26°C) and light-controlled room with a 14-h light/10-h dark photoperiod in accordance with the principles of the Animal Care and Experimentation Committee, Gunma University, Showa Campus. The maternal mice were given food and water ad libitum, and the pups were nursed for 4 d. Eleven-day-old immature mice were killed by cervical dislocation.

**Follicle isolation and culture**

Follicles were mechanically isolated from the ovaries of 11-d-old immature female mice as described (18, 23). Briefly, ovaries were aseptically removed and placed in 15-mm Falcon plastic Petri dishes (FALCON 3037, Becton Dickinson and Co., Rutherford, NJ) containing DMEM (Life Technologies, Inc., Tokyo, Japan) at room temperature. After removing the surrounding tissue, the ovaries were microdissected using two 27-gauge needles attached to 1-ml syringes under a stereomicroscope (Olympus Corp., Tokyo, Japan), and approximately 60 preantral follicles were isolated from each ovary. We cultured type 4 follicles of a diameter of 100–120 μm according to the classification of Pedersen and Peters (24) in a humidified chamber with 5% CO2 in air at 37°C. Type 4 follicles consisted of one to two layers of granulosa cells around the oocyte and an intact basal lamina with theca cells. Histological examination revealed that 86.9% of the sections of these type 4 follicles had at least one thecal cell. This agrees with previous findings that type 4 follicles have 84.5 ± 12.9 granulosa cells per section (18).

Ten type 4 follicles were transferred into 30-mm plastic Falcon Petri dishes containing 1 ml serum-free DMEM supplemented with 6.25 μg/ml of insulin, 6.25 μg/ml of transferrin, 6.25 ng/ml of selenious acid, 5.35 μg/ml of linoleic acid, 0.15% BSA, 15 mM HEPS, 45 μg/ml of penicillin G, 350 μg/ml of streptomycin, and 1.75 μg/ml of Amphotericin B before being cultured in a humidified chamber with 5% CO2 in air at 37°C. Type 4 follicles were cultured with medium alone (control) or with DHEA, DHEAS, T, Δ4, or DHT at concentrations of 1 × 10−7 M or E2 at concentrations of 1 × 10−11 to 1 × 10−7 M and 1 × 10−7 M and 1 × 10−7 M for 4 d without media changes. Next, to ascertain the direct effect of androgens, type 4 follicles were cultured in the presence of T, Δ4, or DHT at concentrations of 1 × 10−7 M in combination with an AR antagonist or an aromatization inhibitor at concentrations of 1 × 10−7 to 1 × 10−5 M. Moreover, to clarify the effect of combined rhFSH and androgens, type 4 follicles were cultured with rhFSH at concentrations of 0.1–100 mIU/ml in the presence of 1 × 10−7 M DHEA, DHEAS, T, Δ4, or DHT at 4 d. Each experiment was repeated at least three to five times. Less than 10% of follicles remarkably degraded during 4 d of culture, and these were removed from the medium.

**Measurement of follicular diameter and hormone assay**

The mean diameter of two-dimensional maximal and minimal lengths in each follicle was measured daily using an inverted microscope (IMT-2, Olympus Corp.). To determine the levels of immunoreactive (IR)-inhibin, E2, and progesterone, the culture medium was collected on day 4 and stored at −20°C. All samples were assayed in duplicate by RIA. IR-inhibin concentrations were measured by double-antibody RIA using rabbit antiserum against bovine follicular fluid inhibin as described (25). E2 levels were determined using an anti-E2 antiserum supplied by Dr. W. F. Crowley, Jr. (26) and estradiol-6-(O-carboxymethyl) oximino-(2-[3H]iodostilbene) (Amersham Pharmacia Biotech, Buckinghamshire, Little Chalfont, UK). The concentrations of progesterone were also measured by double-antibody RIA using a specific rabbit antiserum as described (27). Progesterone-11α-hemisuccinate (2-[3H]iodostilbene) was also purchased from Amersham Pharmacia Biotech. The sensitivity levels of the assays for IR-inhibin, E2, and progesterone were 0.04 ng/ml, 1.00 pg/ml, and 0.01 ng/ml, respectively. The intra- and interassay coefficients of variation for IR-inhibin and E2 were 3.2%, 4.0%, and 2.7% and 2.8%, respectively.

**Assays of cell proliferation in follicles**

Follicular cell proliferation in type 4 follicles cultured with DHEA, DHEAS, T, Δ4, or DHT was evaluated by immunofluorescence staining with 5-bromo-2′-deoxyuridine (BrDU) using a cell proliferation kit (Amersham Pharmacia Biotech). Type 4 follicles were cultured with 1 × 10−7 M DHEA, DHEAS, T, Δ4, or DHT for 4 d and with 1 × 10−5 M BrDU to label proliferative cells for 60 min in a humidified chamber with 5% CO2 in air at 37°C on day 4. After BrDU labeling, cultured follicles were transferred to poly-L-lysine-coated slide glasses, fixed in 3% parafomaldehyde in 0.1 M PBS (pH 7.4) for 30 min at room temperature, solubilized with 1% Triton X-100 in PBS for 10 min, washed in PBS, and incubated in 2% gelatin in PBS for 15 min at 37°C. The follicles were then incubated with sufficient reconstituted nuclease/anti-BrDU in a wet chamber for 120 min at 37°C, washed six times in 0.1% BSA/PBS, and further incubated with FITC-labeled antimoine IgG (Cappel/ICN, Aurora, Ohio) diluted to 1:100 by 1% BSA/PBS for 60 min at 37°C. Nuclei were stained with TOPRO-3 iodide (Molecular Probes, Inc., Eugene, OR) at a 1: 800 dilution. After six washes in 0.1% BSA/PBS, the follicles were mounted as described (28) and examined under a fluorescence microscope (Axioplan, Carl Zeiss, Jena, Germany) and/or a confocal laser scanning microscope (MRC-1024ES, Bio-Rad Laboratories, Inc. Hemel Hempstead, UK). Negative control sections were processed without the labeling reagent (10−5 M BrDU) or reconstituted nuclease/anti-BrDU. The BrDU index was calculated by dividing the number of BrDU-positive cells by the number of BrDU-positive and TOPRO-3-positive cells.

**Statistics**

Results are presented as means ± SEM. One-way ANOVA followed by Scheffe’s multiple comparison was performed to determine statistical differences among groups. A value of P < 0.05 was considered statistically significant.

**Results**

Figure 1 shows changes in the diameter of type 4 follicles cultured with DHEA, DHEAS, T, Δ4, DHT, E1, or E2 at the indicated concentrations for 4 d. The diameter of type 4 follicles increased in a significant dose-dependent manner after stimulation by DHEA, DHEAS, T, Δ4, and DHT but not by E1 or E2. These findings suggested that the stimulatory effect of androgens was direct and not a consequence of being converted to E. The minimum effective dose of each androgen was 1 × 10−10 M, and potency did not significantly differ among the tested androgens. Although all tested androgens increased follicular diameter, IR-inhibin secretion was not significantly increased (data not shown). Androgen administration tended to increase E2 secretion, but the difference was not statistically significant, suggesting that the major role of androgens in type 4 follicles is to cause morphological change rather than functional differentiation.

Figure 2 shows the morphological changes of type 4 follicles cultured for 4 d with medium alone (control) and medium containing DHEA, DHEAS, T, Δ4, or DHT at a concentration of 1 × 10−7 M (Fig. 2A–F). The diameter of follicles cultured in the presence of each of the androgens (Fig. 2B–F) increased remarkably, compared with that of the control (Fig. 2A). Figure 2A–F and Fig. 2A–F′ show BrDU incorporation into granulosa cells. The incorporation of BrDU by follicles cultured in medium containing an androgen significantly increased (Fig. 2B–F′), as did the number of cells.
These findings indicate that androgens stimulate the proliferation of granulosa cells of type 4 follicles. Figure 3 shows the effects of an AR antagonist (OH-Flutamide) and aromatase inhibitor on follicular diameter of type 4 follicles cultured with 10^{-7} M ovarian or adrenal androgens. Although the AR antagonist decreased the diameter of type 4 follicles stimulated by ovarian and adrenal androgens in a dose-related manner, an aromatase inhibitor did not, suggesting that the stimulatory effect of androgens on follicular growth is a direct action.

Figure 4 shows the effect of rhFSH on the growth of type 4 follicles cultured for 4 d in the presence of androgens. Recombinant human FSH alone did not cause any significant change in follicular diameter. However, rhFSH caused a significant and dose-related increase in follicular diameter in conjunction with androgens at a dose of 10^{-7} M. Figure 5 shows IR-inhibin and E2 concentrations in the culture medium. The increase in follicular diameter was accompanied by an increase in IR-inhibin and E2 secretion, suggesting that FSH induced the differentiation of granulosa cells. Although the androgens did not evoke significantly different levels of IR-inhibin secretion, their effects on E2 secretion were significantly different. Among all of the tested androgens, the nonaromatizable androgen DHT was the least potent in evoking E2 secretion.

**Discussion**

Although ovarian androgens have long been implicated as an inhibitor of follicular development (4–6), evidence suggests that they have stimulatory action. Murray et al. (13) showed using an *in vitro* whole-follicle culture system that preantral mouse follicles develop faster in the presence of DHT, DHEAS, T, Δ4, E1, or E2 at concentrations of 1 × 10^{-11} to 1 × 10^{-7} M. CON, Control. Data are presented as means ± SEM. Parentheses represent numbers of follicles. *, P < 0.05, **, P < 0.01, ***, P < 0.0001 vs. control.

Because the response of small preantral follicles from 28-d-old mice is intermediate between immature and adult mice, we speculate that the prepubertal gonadotropin surge is important to the differential responses of immature and adult mice to FSH. The prepubertal gonadotropin surge occurs around 11 d of age and ends by 28 d of age (29). Therefore, an unanswered question is how small preantral follicles of immature mice acquire the initial step in the generation of an FSH response. One putative answer is the presence of intra- and extraovarian growth factors. Because small preantral follicles from 11-d-old mice start to grow as the result of activin A and GH stimulation, we infer that these intra- and extraovarian factors control the initial step of small preantral follicular growth (19). The results of the present study suggest that androgens function in this manner. In particular, adrenal androgens directly stimulate follicular growth, suggesting that the adrenal gland plays an important role in the initiation of prepubertal follicular growth. Despite the fact that the expression and regulation of ARs in neonatal or prepubertal mouse ovaries is not apparent in the adult mouse ovary (30–32), the results of the present study suggest that
FIG. 2. Phase contact images of type 4 follicles cultured with medium alone (control), 1 × 10−7 M DHEA, DHEAS, Δ₄, T, or DHT for 4 d. Photographs A’–F’ represent BrdU-labeled nuclei and photographs A”–F” represent double immunofluorescence images with TOPRO-3 (blue) and BrdU (green). (Magnification, ×400). BrdU index was calculated by dividing the number of BrdU-positive cells by that of BrdU-positive and TOPRO-3-positive cells.
ovarian and adrenal androgens play important roles in granulosa cells of type 4 follicles derived from 11-d-old mice acquiring a response to FSH.

It is well known that the various androgens have different levels of affinity for the AR. Nevertheless, as shown in Fig. 3, the minimal effective dose of androgens was $1 \times 10^{-11}$ to $10^{-9}$ M for both adrenal and ovarian androgens, suggesting that both ovarian and gonadal androgens were equally potent in inducing the growth of type 4 follicles from immature mice. The discrepancy between the different affinity of androgens to their receptor(s) and biological potency can be partly accounted for by the metabolism of androgens during the 4-d culture. It is known that 17β-hydroxysteroid dehydrogenases and 5α reductase, which can convert low-activity sex steroids to more potent forms, are expressed in immature mice ovary (33, 34).

Both ovarian and adrenal androgens stimulated proliferation and increased the number of granulosa cells, which was...
followed by an increase in the size of the follicles. However, the stimulatory effect of androgens on E2 or IR-inhibin secretion from type 4 follicles of immature mice was either relatively weak or absent. We showed that type 4 follicles from 11-d-old mice increase in size and that they secrete more IR-inhibin and E2 upon stimulation by activin A and GH (19). Thus, the action of androgens as seen in the present study is unique. However, Fig. 5 shows that androgen increased IR-inhibin and E2 production from type 4 follicles stimulated by androgens in the presence of FSH. Such synergistic action of androgen with FSH on follicular growth is already known.

In conclusion, both adrenal and ovarian androgens directly stimulate FSH-unresponsive type 4 follicles without producing IR-inhibin and E2 and serve to enhance the sensitivity of FSH responsiveness in type 4 follicles from immature mice. In addition, the present results may answer the question as to how type 4 follicles from immature mice can acquire FSH responsiveness, although biochemical mechanisms are yet to be clarified. In this context, the present results suggest that the adrenal gland plays an important role in the initiation of puberty.

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References

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