Effect of androgens on the development of mouse follicles growing in vitro

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The effects of androgens on ovarian follicular development have been investigated using a whole follicle culture system. Follicles obtained from mouse ovaries and cultured in the presence of anti-androgen serum grew more slowly than control follicles. This effect was reversed by the addition of androstenedione to the medium. A similar effect was obtained when receptor-mediated effects of androgens were blocked using an androgen receptor antagonist. When follicles were grown in concentrations of FSH that are marginal for follicle development, they developed faster in the presence of a non-aromatizable androgen, dihydroxytestosterone. The results indicate that androgens exert a direct, stimulatory role on the growth and development of mouse antral follicles, in vitro.

Introduction

Although gonadotrophins are the primary factors regulating ovarian follicular development, it has become increasingly clear that the effects of FSH and LH are modulated by a variety of paracrine and autocrine factors. The recent discovery of developmentally regulated androgen receptors on the granulosa cells of growing, rat and primate follicles (Tetsuka et al., 1995; Tetsuka and Hillier, 1996; Hillier et al., 1997) indicates that androgens could have direct effects on the development of ovarian follicles, in addition to indirect effects resulting from oestriadiol production after aromatization in the granulosa cells. Spears et al. (1998) showed that aromatizable androgens stimulated follicle development in vitro, whereas oestriadiol had no observable effect, raising the possibility that the stimulatory effect of the androgens is due to its direct action on the follicle.

Studies in vivo have shown contradictory effects of androgens, some of which seemed to show stimulatory effects on follicular development (for example, Hillier and Ross, 1979) while others indicated inhibition (Farookhi, 1985). However, experiments with Tfm/O (testicular feminization) mice, which are unable to respond to androgens, have demonstrated that androgens are not essential for follicular development (Lyon and Glenister, 1974). Studies of ovarian cell lines in vitro have indicated an atretogenic effect of androgens (lia et al., 1985), although cultures of single cell types overlook the potential physiological significance of the influences of other follicular cell types.

We have addressed the question of whether androgens have modulatory effects in promoting follicular development by using a follicle culture system in which individual follicles can be grown from the late preantral to the Graafian stage. The merit of this culture system is that the growth and develop-ment of the follicles in vitro appears to mimic that of maturation in vivo (Gosden et al., 1993; Boland et al., 1993; Spears et al., 1994). The culture system allows the possibility that androgens exert a direct effect on follicular growth or differentiation to be examined in a highly controlled, but none the less physiological environment, while excluding the possibility of confounding extra-ovarian factors.

Materials and Methods

Animals

Twenty-four-day-old C57BL/6 × CBA/Ca F₁ hybrid, female mice were housed in a temperature- and light-controlled room on a 14 h light:10 h dark photoperiod and provided with food and water ad libitum. Female hypogonadal mice (hpg/hpg) produced by mating heterozygous parents were selected on the basis of phenotype and maintained under similar conditions.

Follicle isolation and culture

The mice were killed by cervical dislocation and their ovaries removed to watchglasses containing Leibovitz L-15 medium (Gibco-BRL, Irvine) and 3 mg BSA ml⁻¹ (Fraction V, Sigma, Poole) at 37°C. Preantral mouse follicles (diameter 185 ± 15 µm) were isolated by microdissection using fine needles and pipetted individually to V-wells of microtitre plates in 20 µl α-minimal essential medium (Bibby-Sterilin Ltd, Stone, Staffordshire; Gibco-BRL, Renfrew) under 50 µl mineral oil according to Boland et al. (1993), based on an earlier, similar method (Nayudu and Osborn, 1992). The medium was supplemented with 10 µg human transferrin ml⁻¹ (Sigma) and 5% serum from the hypogonadal mice. Gonadotrophins were added to some

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of the wells at a concentration of either 1 or 5 µIU ml⁻¹. The gonadotrophins used were either human pituitary FSH (NIH-FSH, National Hormone and Pituitary Program, Bethesda, MD), which contained approximately 1% LH as an impurity, or recombinant human FSH (rFSH) (Serono Laboratories, Geneva).

**Measurement of oestradiol**

After transfer of the follicles, a sample of medium from the previous day’s incubation was removed from each well and analysed immunoenzymatically for oestradiol using an ELISA method (Serono Diagnostics, Woking, Surrey). The inter- and intra-assay coefficients of variation were ≤ 5% and the sensitivity was ≤ 18 pmol l⁻¹.

**Growth and morphology of follicles**

Follicular growth was monitored by measuring the diameter of follicles on each day of culture using a precalibrated ocular micrometer at × 40 magnification. Data presented includes measurements from all follicles still viable on the final day of culture. About 20% of follicles burst or became atretic, usually early in the culture period (probably due to damage on the day of dissection). No measurements from such follicles were included in the analyses. No differences were found in the percentage of such follicles across treatments.

In Expt 1, DNA accumulation by preovulatory follicles on day 5 of culture was also assessed using a fluorescent dye, Hoechst 33258 (Sigma) (bisbenzimide: Boland and Gosden, 1994).

The number of preovulatory follicles and the total number of follicles at different antral stages were recorded at the end of each culture period. Preovulatory follicles were classified as having (i) a diameter ≥ 400 µm; (ii) a large antral cavity; (iii) an eccentrically placed cumulus–oocyte complex; (iv) no signs of atresia (dark patches of dead cells within the membrana granulosa). Mid-antral follicles included all follicles with a clearly defined cavity and without signs of atresia, while early-antral follicles exhibited only the initiation of antral development, as viewed under a dissecting microscope.

**Experiment 1: effects of androgen antibody on follicular development**

Anti-androgen antibodies were used to neutralize androgens secreted by cultured follicles or present in the mouse serum. The high-titre antisera was raised by immunizing castrated Suffolk cross-bred sheep with testosterone-3-carboxymethyl-oxime conjugated to egg albumin (Land et al., 1982). There was significant crossreaction of the antibody with androstenedione (0.9%), androsterone (3.3%), dihydroxytestosterone (DHT) (12.1%) and testosterone (100%), but not with either oestrogens or progestagens (Webb et al., 1985).

Thirty preantral follicles were dissected for each experiment and allocated randomly to groups, as follows. Ten follicles were exposed to 3% anti-androgen serum from day 1 to day 5
of culture. A further 10 follicles were cultured with the same serum with an excess of 1 µg androstenedione ml⁻¹ to overwhelm any effects of the antibody. The other 10 follicles were cultured in control medium containing the same percentage of normal sheep serum without androstenedione. The sera had been heat-inactivated for 30 min at 56°C before culture, which leaves antibody activity undiminished, but destroys complement activity. All follicles were cultured in the presence of 1 iu NIH-FSH ml⁻¹.

**Experiment 2: effects of an androgen receptor antagonist**

Follicles were cultured with rFSH (5 iu ml⁻¹) plus dimethyl sulfoxide (DMSO) (1 µl ml⁻¹) alone, or with the addition of 1 x 10⁻⁵ or 1 x 10⁻⁴ mol Casodex 1⁻¹ (bicalutamide: ICI 176,334; Zeneca, Maclesfield, Cheshire), an androgen receptor antagonist, solubilized in 1 µl DMSO ml⁻¹ medium. Follicles were measured and transferred to fresh medium each day. Media were collected for oestradiol analysis on days 3, 4, 5 and 6 of culture, and on the final day of culture (day 6), follicles were scored for stage of antral development.

**Experiment 3: effects of non-aromatizable androgens**

Follicles were cultured in 0.5 iu rFSH ml⁻¹ (marginally adequate for follicle growth) to allow only limited follicular development, in the presence or absence of the non-aromatizable androgen DHT dissolved in 1 µl ethanol ml⁻¹. Control follicles were cultured in 5 iu rFSH ml⁻¹ plus or minus 1 µl ethanol ml⁻¹ medium. Follicles were measured and transferred to fresh medium daily. Media were collected and analysed for oestradiol on days 4, 5 and 6 of culture, and on the final day (day 6) follicles were scored for the stages of antral development.

**Histological preparations**

Cultured follicles were prepared for examination by light microscopy afterfixing overnight in 5% paraformaldehyde. Follicles were then embedded in LR White resin (Taab, Aldermaston) and 2 µm sections were cut and stained with haematoxylin and eosin.

**Statistical analyses**

Each experiment was repeated three times, with the exception of Expt 1 which was performed in duplicate. Probability values (P) between the different groups were determined by the unpaired Student’s t test and analysis of variance.

**Results**

**Experiment 1: effects of anti-androgen antiserum**

There was no significant difference in size between follicles cultured with or without anti-androgen serum during the first 2 days of preantral development (Fig. 1). However, there was a slight, but nevertheless significant, slowing of growth during the later days of culture in the group treated with antiserum (P < 0.05). This trend was reversed when androstenedione was added to the medium. These differences were reflected in the DNA assays. Follicles that were untreated, or received the double supplement, contained 700 ± 30 and 695 ± 26 ng DNA per follicle, respectively, whereas those receiving the antiserum alone contained significantly less (550 ± 23 ng) DNA per follicle (P < 0.02).

Oestradiol production by follicles cultured with the antiserum was substantially reduced on days 3, 4 and 5, when normally it is increasing steeply (P < 0.05). Addition of androstenedione significantly increased oestradiol production above that in control follicles on each of these days (P < 0.01) (Fig. 1).

**Experiment 2: effects of androgen receptor antagonist**

Follicles cultured in the presence of Casodex were significantly less well developed than those cultured in its absence; increasing concentrations of Casodex produced follicles with...
increasingly suppressed growth curves and oestradiol output (Fig. 2); antral cavities at the end of the culture period were also less developed (Fig. 3). The presence of DMSO at low concentrations had no effect on follicular development.

Experiment 3: effects of non-aromatizable androgens

Follicles cultured in low concentrations of FSH grew significantly less well than control follicles cultured with a higher FSH concentration. When DHT was added to the culture medium, follicular growth and antral development were restored to near normal, while oestradiol output remained low (Figs 4, 5 and 6). Ethanol (in which the DHT was solubilized) had no effect on follicle development when added at the same concentration (Fig. 5).

Discussion

The effect of androgens on follicular growth and development has been unclear, with much of the literature contradictory.

Approaching the question from three distinct angles, we have shown in the work presented here that the development of intact mouse follicles in vitro is stimulated by androgens. Spears et al. (1998) found that androstenedione, an aromatizable androgen, stimulated follicular growth as well as oestradiol output in vitro. Although the effect of follicular growth could have been due to the increased oestrogen secretion by these follicles, this interpretation seemed unlikely based on the lack of effect of either oestrogen antibodies or oestrogen receptor antagonists.

In view of the presence of androgen receptors in granulosa cells of growing follicles (Tetsuka et al., 1995), it is quite...
plausible that these steroids have a paracrine action in controlling growth. The experiments described here reveal an effect of androgens on whole ovarian follicles in vitro that was shown to be due, in part at least, to a direct action of the androgens. Follicular development was depressed by the addition of an androgen receptor antagonist, and by an androgen antibody, the latter effect being reversed by the addition of excess androgen, which neutralized the effects of the antibody by competition. Similarly, follicular development was stimulated by the addition of non-aromatizable androgens, confirming a direct effect of androgens rather than via aromatization to oestrogen.

Since antibodies act extracellularly, any ability of antiandrogens to reduce oestradiol production indicates that androgens had been prevented from passing from one cell to another for aromatization to oestrogen. The profound reduction of oestradiol production in vitro, therefore, substantiates the two-cell theory for the mouse and is in agreement with comparable studies that have been carried out in vitro in other species (Baird, 1977) and in vitro using co-cultures of granulosa and theca–interstitial cells (Lui and Hsueh, 1986). The present work showed evidence of an intrafollicular role of androgens. Antibodies to these steroids produced a slower rate of follicle growth during the early stages of Graafian development, which was reversible with androstenedione supplementation. Although oestrogen concentrations were reduced in these experiments, the results of Spears et al. (1998) indicate that it is unlikely that oestrogen is responsible for these effects on follicle growth.

The androgen-receptor antagonist, Casodex, inhibits the ability of androgens to influence follicle development via receptor binding, without affecting aromatization to oestrogen, or altering the potential for oestrogen to alter follicle development. Follicles treated with increasing doses of Casodex exhibited increasing inhibition of follicular development and oestrogen secretion. Follicles treated with Casodex had greatly diminished antral development by the end of the culture period, and oestradiol output was commensurate with their retarded development. This effect of Casodex appears to be due to arrested development, rather than a reduced rate of development, as follicles cultured for an additional 2 or 3 days were still unable to develop to the Graafian stage (A. A. Murray, R. G. Gosden, V. Allison and N. Spears, unpublished results).

In the final experiment reported here, follicular development was retarded by culturing the follicles in reduced concentrations of FSH. A concentration of FSH was chosen that supported follicle survival and growth, but that did not promote development to the Graafian stage within the usual culture period. The inadequate development of follicles cultured in the low FSH concentrations could be partially overcome by the addition of a non-aromatizable androgen, DHT, although androgens were unable to stimulate follicular development in the complete absence of FSH (A. A. Murray, R. G. Gosden, V. Allison and N. Spears, unpublished results), presumably due to the vital survival effect of FSH (Chun et al., 1996; Spears et al., 1998). In this, and the first experiment, in which androgens were added to the culture medium, concentrations were chosen that reflect concentrations in both human and pig follicular fluid (Ding and Foelix, 1992; McNatty et al., 1979) since no comparable data are available for rodents.

Tettsuka and Hiller (1996) reported the ability of DHT, in combination with FSH, to stimulate P450 aromatase and the production of the mRNA encoding the androgen receptor. Gherschelich et al. (1994) have also shown that DHT enhances the stimulatory effect of FSH on the production of 17ß-hydroxysteroid-dehydrogenase by granulosa cells, while the ability of androgens to stimulate follicular development in the presence of low concentrations of oestrogens has been shown in rhesus monkeys using an aromatase inhibitor (Zelinsky-Wooten et al., 1993). It is of interest that, in the present experiments, oestrogen concentrations did not increase propor-
tionately with follicular development in the follicles cultured in the presence of DHT; this is possibly due to the ability of DHT to act as a competitive inhibitor to aromatase, which is known to occur in vitro (Hillier et al., 1980).

The expression of androgen receptors has been shown to be developmentally regulated, with the number of receptors highest in preantral and early antral follicles. There is then an FSH-dependent decrease in numbers of receptors during pre-ovulatory growth (Hillier et al., 1997; Tetsuka and Hillier, 1996), and it is probable that the stimulatory effects of androgens on follicular development are, similarly, transient.

The stimulatory effects of androgens on ovarian development have been known for some time (for example, Schomberg et al., 1976; Hillier and Ross, 1979), but they were often considered to be due to indirect stimulation, via oestrogen. As a result of this possibility, and due to the clear effects of oestrogens in stimulating ovarian development in hypophysectomized rats (Williams, 1940), oestrogens are frequently assumed to stimulate follicular development, whereas androgens are often cited as atretogenic. However, there is now mounting evidence that, in many species, including the mouse, there is no simple causal relationship between the oestrogen–androgen balance within a follicle and developmental fate. Zelinski-Wootten et al. (1993) showed that high androgen: oestrogen ratios in rhesus monkeys do not inhibit follicular development, although fertilization rates of the resulting oocytes were decreased. Oestrogen appears to have little effect on the development of mouse follicles (see Spears et al., 1998), and in the work presented here, we demonstrate a stimulatory role for murine androgens within the developing follicle. It remains to be explained whether the inhibitory effects of androgens shown by others both in vitro (Jia et al., 1985) and in vivo (Billig et al., 1993; Farookhi, 1985; Payne et al., 1956) reflect species differences or extra-ovarian or culture phenomena.

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