Location and developmental regulation of androgen receptor in primate ovary

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Locally produced androgens act via granulosa cell androgen receptors to modulate follicular responsiveness to gonadotrophins and thereby contribute to the paracrine regulation of ovarian function. We used quantitative androgen receptor immunocytochemistry to assess androgen receptor distribution in relation to pre-ovulatory follicular development in the common marmoset (Callithrix jacchus), a New World primate that ovulates two to four follicles in each ~28 day ovarian cycle. Ovaries from four adult females in the late follicular phase and from four in the luteal phase were fixed in 4% paraformaldehyde and subjected to an immunocytochemical analysis using a polyclonal androgen receptor antibody with detection by a standard avidin–biotin–peroxidase technique for alkaline phosphatase. Specific androgen receptor immunostaining occurred mainly in granulosa cell nuclei, with little or no specific staining in theca, stroma or oocytes. Granulosa cell androgen receptor immunostaining was most abundant in healthy preantral/early antral follicles, being low or absent from pre-ovulatory follicles and corpora lutea. Differences in granulosa cell androgen receptor immunostaining between immature (0.1–1.0 mm diameter) and pre-ovulatory (≥ 2.0 mm diameter) follicles were quantified using a videodensitometric analysis of grey-scale values. Readings were taken from the granulosa cell layers of 53 immature follicles and 10 pre-ovulatory follicles in late follicular phase ovaries. The average androgen receptor level in granulosa cells of immature follicles proved to be 4.2-fold higher (P < 0.01) than that in granulosa cells of pre-ovulatory follicles. Because other evidence suggests that paracrine androgen action in granulosa cells converts from stimulation to inhibition as follicles mature, we speculate that a development-related reduction in androgen receptor numbers serves to ‘protect’ granulosa cells against the inhibitory action of androgen, thereby promoting pre-ovulatory follicular dominance in primate ovarian cycles. Key words: androgen/atroresia/follicular development/gonadotrophins/granulosa cells

Introduction
The trophic action of follicle stimulating hormone (FSH) on granulosa cells in ovarian follicles is subject to modulation by locally produced steroidal and non-steroidal factors (Hillier, 1994; Richards, 1994). A major influence on granulosa cell function is exerted by androgens produced by luteinizing hormone (LH)-stimulated theca cells (Daniel and Armstrong, 1980; Hillier and de Zwart, 1981). In rat ovary, granulosa cells possess androgen receptors which bind androgen (Schreiber et al., 1976; Schreiber and Ross, 1977) and modulate cAMP-responsive gene expression (Hillier and de Zwart, 1982; Fitzpatrick and Richards, 1991). Recently it was reported that granulosa cell androgen receptor mRNA and protein are down-regulated during FSH-induced pre-ovulatory follicular development in the rat, suggesting that paracrine androgen action is influenced by development-related changes in granulosa cell androgen receptor expression (Tetsuka et al., 1995; Tetsuka and Hillier, 1996).

Androgens are also implicated in the local control of human ovarian function. Although it has been established that granulosa cells in human (Horie et al., 1992) and non-human (Hild-Petito et al., 1991) primate ovaries express androgen receptor, it is not known if the granulosa cell androgen receptor system in primates is subject to developmental regulation. This study was therefore designed to localize androgen receptor protein by immunocytochemistry and to determine if granulosa cell androgen receptor expression is regulated during follicular development using the common marmoset (Callithrix jacchus) as a laboratory primate model (Hillier et al., 1987).

Materials and methods

Animals
The primate model selected was the common marmoset monkey, a New World monkey with an ~28 day-long ovarian cycle that typically ovulates two to four follicles per cycle (Hillier et al., 1987). Adult females were from the breeding colony maintained at the Medical Research Council Reproductive Biology Unit Primate Centre, Edinburgh, UK. The study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Blood samples were collected on alternate days by femoral venepuncture without anaesthesia to establish normal ova-luteal cycles. Following centrifugation for 20 min at 1000 g, separated plasma was stored at –20°C until required for progesterone assay. Criteria for the occurrence of ovulation and normal luteal phase length (18–20 days) were as described previously (Smith et al., 1990). Four animals were in the luteal phase, confirmed by the presence of at least two recently formed corpora lutea. Accurately staged late follicular phase tissue was obtained from four other animals 8–9 days after inducing luteal regression with a single i.m. injection of 0.5 µg synthetic prostaglandin F2α analogue (Estrumate™; ICI-Zeneca, Macclesfield, UK) given on day 14 of the previous luteal phase (Summers et al., 1985).

Animals were sedated using ketamine hydrochloride (Parke-Davis...
Veterinary, Pontypool, UK) i.m. and killed with an i.v. injection of 400 µl Euthetal™ (sodium pentobarbitone; Rhone Merieux, Eire). Both ovaries were removed immediately and fixed overnight in a solution of 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Pituitary and other tissues were also removed for use in experiments to be described elsewhere.

**Immunocytochemistry**

Fixed ovaries were processed through an automatic tissue processor for 16 h and embedded in paraffin wax. Paraffin wax sections (5 µm) were mounted on slides coated with 3-amino-propyltriethoxyxilane (Sigma Chemical Co. Ltd, Poole, UK) and incubated overnight at 55°C so that the sections adhered to the slide. Paraffin wax was removed by immersion in xylene, and the sections were rehydrated by a decreasing alcohol series. Sections were then subjected to a 10% formic acid treatment for 1 h and incubated in 1 M sodium citrate buffer (pH 6.0) for 1 h. Specific androgen receptor immunostaining was located mainly in granulosa cell androgen receptor numbers are developmentally regulated, being most abundant in immature androgens (Nevalainen et al., 1991) and human (Horie et al., 1992) ovaries.

**Results**

Specific androgen receptor immunostaining was located mainly in granulosa cell nuclei with minimal staining in theca, stroma, oocytes or the surface epithelium. Staining was most intense in the granulosa cells of apparently healthy early antral follicles. Follicles at advanced stages of atresia showed relatively light androgen receptor staining (Figure 1).

Androgen receptor immunostaining was relatively weak in the granulosa cells of pre-ovulatory follicles compared with those of less mature follicles in the same ovaries (Figure 1A–C). Granulosa cells of healthy, immature follicles in luteal phase ovaries also showed intense androgen receptor immunostaining (Figure 1D–F). Specific androgen receptor immunostaining in luteal and interstitial cells was minimal in comparison with the granulosa cells of immature follicles in the same ovaries (Figure 1F).

A quantitative image analysis confirmed that within individual animals the amount of androgen receptor immunostaining of granulosa cells in pre-ovulatory follicles was 2- to 11-fold lower than in immature follicles (Figure 2). Combining the data from all four late follicular phase animals revealed an average CPV value (mean ± SE) of 13.4 ± 0.9 (n = 10) for pre-ovulatory follicles versus 55.1 ± 2.2 (n = 53) for immature follicles (P < 0.01).

**Discussion**

These results show that during pre-ovulatory follicular development granulosa cell androgen receptor immunostaining declines in common marmoset ovaries, just as in rat ovaries (Tetsuka et al., 1995). In the late follicular phase, granulosa cell androgen receptor immunostaining in marmoset pre-ovulatory follicles was markedly suppressed relative to granulosa cells in immature follicles in the same ovaries, and remained low or absent in corpora lutea during the luteal phase. This suggests that granulosa cell androgen receptor numbers are developmentally regulated, being most abundant in immature preantral–early antral follicles and declining throughout pre-ovulatory follicular development in response to stimulation by FSH.

The present clear-cut findings contrast with the results of earlier studies using immunocytochemistry to locate androgen receptor in primate ovaries. Studies of rhesus monkey (Hild-Petito et al., 1991) and human (Horie et al., 1992) ovaries...
Androgen receptor in primate ovary

Figure 1. Immunocytochemical localization of androgen receptor in common marmoset ovaries. (A) Section from a late follicular phase ovary stained with androgen receptor antiserum. Note the reduced level of specific staining in granulosa cells (arrow heads) of a pre-ovulatory follicle (po) compared with two apparently healthy immature follicles (if). Granulosa cells in an atretic follicle (af) also show minimal specific staining (original magnification ×100). (B) Section from same ovary stained with non-immune serum as a control for non-specific immunostaining (original magnification ×100). (C) Section from a different late follicular phase ovary stained with androgen receptor antiserum, again showing relatively light androgen receptor immunostaining in the granulosa cells (arrow heads) of a pre-ovulatory follicle (po) compared with granulosa cells in an atretic follicle (af) (original magnification ×40). (D) Close up of the same section revealing the absence of specific androgen receptor immunostaining in oocyte and theca (original magnification ×200). (F) Section from an early luteal phase ovary showing a corpus luteum (cl) with light androgen receptor immunostaining relative to the granulosa cells in an immature follicle (if) in the same section (right hand) (original magnification ×100).

have indicated specific androgen receptor staining in several ovarian cell types — including granulosa and luteal cells — but differences in relation to follicular maturity have not been noted previously. The ease and certainty with which pre-ovulatory follicles can be recognized in this primate species (Hillier et al., 1987) presumably contributes to the clarity of these results. Interspecies differences might exist in ovarian androgen receptor location and expression, even between primates. However, the most likely explanation is methodological. Not only did we use a highly specific androgen receptor antiserum to a synthetic peptide representing a continuous epitopic site near the N-terminal end of the human androgen receptor (Nevalainen et al., 1991), but a microwave ‘antigen retrieval’ step was also included prior to the incubation of tissue sections with the androgen receptor antiserum. We have found this step to be crucial for the detection of specific androgen receptor immunostaining in the marmoset ovary, similar to our experience with the rat ovary (Tetsuka et al., 1995).

Loss of granulosa cell androgen receptor immunostaining during pre-ovulatory development could be due to a reduction in the level of androgen receptor protein present and/or to a change in androgen receptor immunoreactivity. In the rat ovary, pre-ovulatory development induced by treatment with FSH is associated with down-regulation of androgen receptor mRNA (Tetsuka et al., 1995; Tetsuka and Hillier, 1996), indicating that the granulosa cell androgen receptor gene is negatively regulated by FSH and/or local regulatory factors produced in response to the trophic action of FSH. It remains to be determined if a similar decline in androgen receptor mRNA expression occurs during pre-ovulatory follicular development in primates.
Figure 2. Quantitative androgen receptor immunostaining of granulosa cells in common marmoset monkey ovaries obtained during the late follicular phase of the ovarian cycle. One section from each ovary was taken for videodensitometric analysis following staining with androgen receptor antiserum, with correction for non-specific staining, as described in the text. All apparently healthy follicles containing at least two layers of granulosa cells were included in the analysis. Specific androgen receptor immunostaining, expressed as ‘corrected pixel value’ (mean ± SE, n = 5), is shown for the two to four pre-ovulatory follicles (>2.0 mm diameter; open bars) and up to 15 randomly numbered immature (<1.0 mm diameter; hatched bars) follicles in each pair of ovaries from four animals. Note that pre-ovulatory follicles consistently showed lower (*, P < 0.01) amounts of androgen receptor staining than immature follicles.

The high number of androgen receptors present in the granulosa cells of preantral–early antral follicles seems likely to be of physiological relevance. Locally produced steroidal and non-steroidal factors, including androgens, are thought to modulate FSH responsiveness at intermediate stages of follicular development, thereby contributing to the mechanism that determines which follicles are ‘selected’ to achieve full pre-ovulatory maturity (Hillier et al., 1988).

The functional relevance of androgen receptor loss in pre-ovulatory follicles is less obvious. It has been shown previously that the mode of androgenic modulation of FSH action on marmoset granulosa cells in vitro switches from stimulatory to inhibitory as follicular development advances (Harlow et al., 1986, 1988; Shaw et al., 1989). High intrafollicular levels of androgen are not by themselves a marker of follicular atresia, but the intrafollicular hormonal milieu in large (>6 mm diameter) antral follicles is dominated by androgens when they are atretic as opposed to oestrogen when they are ‘healthy’ (Westergaard et al., 1986). Thus a decline in granulosa cell androgen receptor numbers during advanced pre-ovulatory development might serve to diminish the potentially deleterious effects of androgen on granulosa function, forming part of the intra-ovarian mechanism that determines which follicles become dominant and ovulate in primate ovarian cycles.

Present data do not rule out androgen receptor-mediated androgen action at non-follicular ovarian sites. Consistent with previous reports (Hild-Petito et al., 1991; Horie et al., 1992), scattered luteal cells and occasional stromal interstitial cells also showed specific immunostaining for androgen receptor in marmoset ovaries, albeit at much lower levels than in granulosa cells.

In conclusion, androgen receptor immunoreactivity is abundant in the granulosa cells of the common marmoset ovary. Androgen receptor immunostaining is strongest in the granu-
loosa cells of preantral–early antral follicles and declines during pre-ovulatory follicular development. Because androgens have an established paracrine role in folliculogenesis, the down-regulation of granulosa cell androgen receptor is likely to be of physiological relevance to the gonadotrophic control of ovarian function in primates.

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References


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