Immunolocalization of aromatase P-450 in ovarian tissue from pregnant and nonpregnant mares and in ovarian tumours

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Aromatase P-450 (P-450_{arom}) is a crucial regulatory enzyme that is necessary for conversion of androgens to oestrogens. Corpora lutea and follicles were obtained from the ovaries of cyclic mares and from mares at day 20 and days 40–70 of pregnancy. The presence of P-450_{arom} within specific cell types was investigated by immunostaining to determine potential sites of oestrogen synthesis. Immunoreactivity for P-450_{arom} was confined to the granulosa layer of non-atretic follicles > 5 mm in diameter and to corpora lutea at all stages of the oestrous cycle and during pregnancy. These findings confirm that aromatization of androgens occurs within the granulosa cells of the preovulatory follicle of the mare and that the corpus luteum of the mare has the capacity for oestrogen production if adequate androgen substrate is available. Granulosa cells in ovarian tissue from three mares with granulosa cell tumours showed little staining for P-450_{arom}, which suggests that these tumours have little aromatizing capacity.

Introduction

The mechanism of steroidogenesis by follicles and corpora lutea in mares remains contentious. Originally it was reported that the primary steroidogenic role of granulosa cells in the preovulatory follicle was to produce progesterone, while the theca interna was responsible for oestradiol production (Short, 1962; Channing and Grieves, 1969; Younglai, 1971). The corpus luteum was not thought to produce oestrogen (Short, 1964; Mahajan and Samuels, 1974). However, it was also found that, although granulosa and luteal cells had only a limited capacity to produce oestradiol *de novo*, they could aromatize androgens to oestradiol *in vitro* (Ryan and Short, 1965; Mahajan and Samuels, 1974; Al-Timimi *et al.*, 1989). Interaction between cultured granulosa and theca cells from mare follicles was found to be necessary for oestrogen synthesis, and it was then suggested that androgens of thecal origin were aromatized to oestradiol by granulosa cells (Sirois *et al.*, 1991). Although the corpus luteum of the nonpregnant mare is not thought to produce oestrogen (Short, 1964; Mahajan and Samuels, 1974), recent studies *in vivo* have provided evidence that corpora lutea from mares that are between 35 and 70 days of pregnancy act as a source of oestrogen (Daels *et al.*, 1991).

Granulosa cell tumour is the most common neoplasm of the mare ovary and represents 2.5% of all equine neoplasms (Sundberg, 1977). These neoplasms are frequently accompanied by behavioural abnormalities that are manifest as anoestrus, nymphomania or stallion-like behaviour. Testosterone concentrations are often increased in mares exhibiting aggressive male-type behaviour and comprise approximately 50% of

affected mares (Stabenfeldt *et al.*, 1979). However, concentrations of oestradiol in mares with granulosa cell tumours are variable and are often not related to the behavioural changes (Stabenfeldt *et al.*, 1979). It has been proposed that aromatization of testosterone to oestradiol may be low in affected mares, as mares with very high concentrations of circulating testosterone do not necessarily have concomitantly high oestradiol concentrations (Stabenfeldt *et al.*, 1979). However no studies have determined the aromatase activity of equine granulosa cell tumours.

The development of antibodies against various steroidogenic enzymes has allowed detailed examination of ovarian steroidogenesis in other species, but to date there have been no studies in the mare ovary on the expression of steroidogenic enzymes at the cellular level throughout the oestrous cycle and pregnancy. In the ovarian steroidogenic pathway, aromatase cytochrome P-450 (P-450_{arom}) is a crucial regulatory enzyme that is necessary for conversion of androgens to oestrogens. In the present study, ovarian tissue was obtained from mares at various stages of the oestrous cycle, during early pregnancy, and from three mares with granulosa cell tumours, and stained immunohistochemically to detect and localize the cells capable of aromatization.

Materials and Methods

Follicles and corpora lutea were obtained from 20 pony mares aged 4–18 years and weighing 250–380 kg. The stage of cycle was monitored by daily ultrasonographic examination of the ovaries and uterus. Day of ovulation was designated day 0. Ovaries were removed by a colpotomy incision as described by Watson and Sertich (1990). Neuroleptanalgesia was induced

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by intravenous administration of romifidine ($50 \mu\text{g kg}^{-1}$; Sedivet, Boehringer Ingelheim Ltd, Bracknell, Berks) and butorphanol ($25 \mu\text{g kg}^{-1}$; Torbugesic, Willows Francis Veterinary Ltd, Crawley, Surrey). Flunixin meglumine (1 mg kg^{-1} ; Finadyne, Schering-Plough Animal Health Ltd, Watford, Herts) was administered immediately after surgery. Corpora lutea were obtained from between two and six mares in early (days 2–3), and mid- (days 7–12) dioestrus, in the early follicular phase (day 16), 1 day after administration of the prostaglandin $\text{F}_{2\alpha}$ analogue cloprostenol ($263 \mu\text{g}$; Estrumate, Mallinckrodt Veterinary Ltd, Uxbridge, Middlesex) in mid-dioestrus, and during pregnancy (days 20 and days 40–70). Preovulatory follicles were obtained during oestrus ($n = 7$) and smaller follicles were dissected out of the ovary during dioestrus ($n = 5$). A jugular blood sample was collected from all mares before surgery and the plasma was stored at -20°C until assayed for progesterone. Granulosa cell tumours were obtained from a further three mares by midline laparotomy under general anaesthesia. Serum concentrations of testosterone and oestradiol were determined by a commercial laboratory (SCL Bioscience Services Ltd, Cambridge) in samples taken before surgery from these three mares.

Tissue was embedded in OCT compound (Miles Inc., Elkhart, IN) on cork disks and snap frozen in a slurry of isopentane–dry ice. The samples were then stored at -70°C .

Immunostaining procedures

Sections ($6 \mu\text{m}$) were immunostained using an avidin–biotin complex method described by Watson and Thomson (1996). The primary antibody, which was kindly provided by E. Simpson (University of Texas), was raised in rabbits against synthetic aromatase cytochrome P-450, and used at a final concentration of $50 \mu\text{g ml}^{-1}$. The chromagen used (AEC; 3-amino-9-ethylcarbazole) produced a red reaction product and the sections were counterstained with Meyer's haematoxylin.

Negative control sections in which the first antibody was replaced with normal horse serum, and positive controls of horse testis, were included with each batch of samples.

Assay of plasma progesterone

Progesterone concentrations were determined in unextracted plasma using a radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA) employing an ^{125}I -progesterone tracer (Watson *et al.*, 1995). The limit of detection of the standard curve was 0.25 ng ml^{-1} and the inter- and intra-assay coefficients of variation were 6.0 and 6.8%, respectively.

Results

Circulating progesterone concentrations in all mares were $> 1 \text{ ng ml}^{-1}$ except for mares in early follicular phase and on the day after treatment with prostaglandin $\text{F}_{2\alpha}$.

Immunoreactivity for P-450_{arom} was confined to the granulosa cells of non-atretic follicles $> 5 \text{ mm}$ in diameter (Fig. 1). The granulosa cells of small follicles $< 5 \text{ mm}$ in diameter and the lining of atretic follicles did not stain. No staining for

Fig. 1. Immunoreactivity of P-450_{arom} in a 30 mm preovulatory follicle taken from a mare's ovary. Note localization of staining in granulosa cells (arrow). Sections were counterstained with Meyer's haematoxylin. Scale bar represents $10 \mu\text{m}$.

P-450_{arom} was detected in the thecal cells, stromal cells or preantral follicles.

The early corpus luteum showed many strongly staining luteinizing granulosa cells (Fig. 2a). After structural reorganization of the corpus luteum in mid-dioestrus, there was overall weak immunoreactivity with patchy, strongly stained large cells (Fig. 2b). Cells within the trabeculae did not show staining. On day 16, when the corpora lutea started to show structural changes associated with luteolysis, they still retained the same staining pattern as in mid-dioestrus. This pattern also persisted in corpora lutea obtained 24 h after administration of cloprostenol. These staining patterns were consistently present in replicate sections stained on different days.

On day 20 of pregnancy, uniformly pale immunostaining was present. Dark-staining cells were not apparent (Fig. 3a), but by days 40–70 dark staining cells were again obvious (Fig. 3b). In one secondary corpus luteum removed on day 50 that had ovulated 10 days previously, patches of darkly staining non-luteinized granulosa cells were seen.

Two of the mares with granulosa cell tumours had low testosterone and oestrogen concentrations (testosterone = 0.28 and 0.06 nmol l^{-1} , oestradiol = $< 20 \text{ pmol l}^{-1}$) and one had increased concentrations (testosterone = 1.0 nmol l^{-1} , oestradiol = 230 pmol l^{-1}). Granulosa cells from the tumours of the mares with low testosterone showed very little staining for P-450_{arom}. Only occasional individual cells were strongly positive (Fig. 4). In the mare with increased concentrations of blood testosterone and oestradiol, occasional groups of granulosa cells showed positive staining.

In sections in which the primary antibody was excluded, there was no evidence of endogenous peroxidase activity (Fig. 5a). In positive controls (testis), interstitial cells stained, whereas no staining was observed in the seminiferous tubules (Fig. 5b).

Discussion

This is the first report of immunolocalization of P-450_{arom} in equine ovarian tissue. A homologous antibody for equine P-450_{arom} was not available, but aromatase is known to have

Fig. 2. Immunoreactivity of P-450_{αrom} in (a) the corpus luteum taken from the ovary of a mare at day 2 of the oestrous cycle. Luteinizing granulosa cells are strongly stained (arrows); and (b) corpus luteum at day 10 of the oestrous cycle. Note the patchy, strong staining localized to individual cells (arrows). Sections were counterstained with Meyer's haematoxylin. Scale bars represent 10 μm.

Fig. 3. Immunoreactivity of P-450_{αrom} in (a) the corpus luteum taken from the ovary of a mare at day 20 of pregnancy. The large cells show uniform weak immunostaining. Note the unstained trabecula (arrow). (b) The corpus luteum at day 70 of pregnancy. Individual large cells are strongly stained. Sections are counterstained with Meyer's haematoxylin. Scale bars represent 10 μm.

wide sequence homology among different species (Simpson *et al.*, 1994) and a heterologous antibody has previously been found to recognize interstitial cells in stallion testes (Eisenhauer *et al.*, 1995). In fact, stallion testis was used as the positive control in our study and staining was confined to interstitial cells as previously reported (Eisenhauer *et al.*, 1995).

Immunoreactivity to P-450_{αrom} was present in the granulosa cells of medium to large ovarian follicles but was absent in small follicles (< 5 mm). Measurement of steroid concentrations in follicular fluid from mares has shown that highest concentrations are present in large vascularized follicles, with only low concentrations present in small follicles (Short, 1961; Van Rensburg and Van Niekerk, 1968; Channing and Grieves, 1969). This finding is in agreement with the lack of enzyme immunoreactivity in small follicles in our study. Atretic follicles, in which the separate identity of the theca and granulosa cells was lost, showed no evidence of immunoreactive P-450_{αrom}. Concentrations of oestrogen are very low in these follicles (Kenney *et al.*, 1979). Results from cell culture experiments and analysis of steroid content of follicular fluid have led to the conclusion that the theca interna is responsible for oestrogen synthesis in the preovulatory follicle of the mare (Short, 1962; Channing and Grieves, 1969; Younglai, 1971; Hay *et al.*, 1975). However more recent studies have suggested

Fig. 4. Immunoreactivity of aromatase P-450 in granulosa cells taken from the ovary of a mare with a granulosa cell tumour. Only a few cells showed strong positive staining (arrows). Scale bar represents 10 μm.

that testosterone is the main product of cultured equine thecal cells, and that androgens of thecal origin may be aromatized to oestradiol by granulosa cells (Sirois *et al.*, 1991; Tucker *et al.*,

Fig. 5. (a) Negative control section of corpus luteum from the ovary of a mare in which the primary antibody for aromatase P-450 was excluded. (b) Positive control section of stallion testis. No immunostaining for aromatase P-450 was present in the seminiferous tubules, whereas the surrounding interstitial cells stained positive. Scale bars represent 10 μ m.

1991). Culture of isolated cell types can be subject to contamination with other cell populations (Ryan and Short, 1965) and to changes that can transform cells *in vitro* such as the propensity for granulosa cells to luteinize *in vitro* (Channing, 1969). *In situ* detection of enzyme by immunohistochemical staining does not allow quantitation of enzyme content, but does localize the enzymes to specific cell types. The results of the present study would indicate, therefore, that aromatization of androgens is confined to granulosa cells within the follicle.

Most studies agree that oestradiol is not present in the corpus luteum of the nonpregnant mare (Short, 1964; Mahajan and Samuels, 1974); however, luteal cells and microsomes from corpora lutea appeared to be capable of aromatizing testosterone and androstenedione when these were provided as substrates (Mahajan and Samuels, 1974; Al-Timimi *et al.*, 1989). Furthermore, the presence of oestradiol in the mid-luteal corpus luteum was reported by Younglai (1971) and there is unpublished circumstantial evidence that oestrogen secretion increases during the mid-luteal phase and decreases rapidly after administration of PGF_{2 α} (cited in Daels *et al.*, 1991). We have shown that immunoreactive P-450_{arom} is present in luteal cells throughout dioestrus. Therefore, the potential for oestrogen production resides within the corpus luteum of the cyclic

mare. Although thecal cells are present in the trabeculae of the mare corpus luteum (Harrison, 1946), and small luteal cells that might be expected to be of thecal origin are steroidogenic (Kelly *et al.*, 1988; Broadley *et al.*, 1994), the small thecal-derived cells are vastly outnumbered by the large granulosa-derived cells. As the granulosa-derived cells appear to depend on the theca-derived cells for androgen substrate, the ability of the corpus luteum to produce androgens and oestrogens may be directly related to the extent to which thecal cells contribute to the corpus luteum (Henderson and Swanston, 1978). However, there appear to be marked differences in steroidogenic capacities of ovarian cells among species (Hammerstein *et al.*, 1964; Watson and Leask, 1975; Al-Timimi *et al.*, 1989; Lautincik *et al.*, 1994). Most studies have shown that the corpus luteum of the non-pregnant mare does not produce oestrogen. If this is the case, high concentrations of circulating progesterone during dioestrus could inhibit aromatase activity in the mare ovary (Amri *et al.*, 1993) as has been proposed in sows (Gregoraszcuk, 1994) and rats (Fortune and Vincent, 1983). Therefore, factors other than cell type may influence luteal oestrogen production.

In the mare corpus luteum, immunostaining for P-450_{arom} was present shortly after luteolysis, whereas the human corpus luteum demonstrates neither P-450_{arom} immunoreactivity nor mRNA expression after luteal regression (Suzuki *et al.*, 1993). However the corpora lutea examined by Suzuki *et al.* (1993) were probably older than the mare corpora lutea in the study reported here. The presence of immunoreactivity for aromatase in the mare corpora lutea in the period immediately after luteolysis, when steroid production ceases, suggests that cessation of steroidogenesis is dependent on mechanisms other than disappearance of aromatase. It is thought that PGF_{2 α} may cause luteolysis in domestic species by inhibition of second messenger pathways (Fletcher and Niswender, 1982) rather than by having a direct effect on steroidogenic enzymes.

The mare corpus luteum appears to comprise at least two distinct populations of large luteal cells. Some individual cells stained very heavily for aromatase, whereas the staining in surrounding cells was much weaker. Apparent differences in staining intensities within cell populations have similarly been reported in granulosa cells of preovulatory follicles of rats and mice (Ishimura *et al.*, 1989), interstitial cells in stallion testes (Eisenhauer *et al.*, 1995), and in theca interna cells in follicles of sheep and cattle (Conley *et al.*, 1995), leading to suggestions that there may be functional differences within these populations. Although immunostaining is not a quantitative technique, a significant correlation has been shown between immunointensity for aromatase P-450, measured by computerized image analysis, and enzyme activity (Sasano, 1994). During early pregnancy the difference in staining between cells in the mare corpus luteum was less marked, whereas by day 40, strong areas of staining had returned.

It seems likely that equine chorionic gonadotrophin (eCG), the concentration of which increases around day 35 of pregnancy, acts as the trigger for oestrogen production by the primary corpus luteum. Recent work has shown that treatment with eCG stimulates oestrogen secretion in pregnant mares with a corpus luteum, but not in those in which the corpus luteum has been removed (Daels and Albrecht, 1995). Luteal mRNA encoding aromatase P-450 in the mare corpus luteum

was lower after the onset of eCG secretion than at any other stage during pregnancy, despite the increased oestrogen secretion (Albrecht *et al.*, 1995). This observation led to the suggestion that the increase in luteal oestrogen secretion is due to post-transcriptional regulation of aromatase activity by eCG (Albrecht *et al.*, 1995). However, there is usually a good correlation between concentrations of mRNA encoding aromatase P-450 and amount of enzyme protein (Doody *et al.*, 1990). The results reported here show the appearance of darker staining in certain individual cells after the onset of eCG secretion, which might indicate that these cells were producing more aromatase than earlier in pregnancy, but the pattern and intensity of staining was not different from that shown by corpora lutea from non-pregnant mares. These data suggest that P-450_{17 α} activity, which provides the androgen substrate, may be the important limiting factor in secretion of oestrogen by the corpus luteum of pregnant mares. The appearance of P-450_{17 α} in luteal cells may be stimulated by eCG. Indeed P-450_{17 α} has been induced in thecal cells of immature rats by treatment with eCG and hCG (Ishimura *et al.*, 1990), and injection of rats with eCG increased total hybridizable transcript for P-450_{17 α} (Doody *et al.*, 1991). We have preliminary evidence that P-450_{17 α} is present in the corpus luteum during pregnancy but not during the oestrous cycle (Rodger *et al.*, 1995). Similarly a previous report has described low 17,20 lyase activity of corpora lutea from non-pregnant mares as demonstrated by the poor capacity of luteal tissue to metabolize progesterone to androgens (Mahajan and Samuels, 1974), although another study showed that low concentrations of androgens were produced (Al-Timimi *et al.*, 1989).

The biological significance of any oestrogen secretion by the primary corpus luteum is questionable as it is well documented that mares ovariectomized on day 34 or 35 of pregnancy and supplemented with only progestagens can maintain a pregnancy until placental steroid production takes over at around day 100 (Shideler *et al.*, 1982).

Ovarian tumours are relatively common in mares and account for approximately 5.6% of all neoplasms (Righ *et al.*, 1985). By far the commonest type of ovarian neoplasm is the granulosa cell tumour (McEntee, 1990). The failure of the tissue from two of the granulosa cell tumours to show significant immunostaining and the low level of staining in the other is interesting and is similar to immunostaining of human granulosa cell tumours (Sasano, 1994). The high circulating oestrogen concentrations present in some women with granulosa cell tumours (Besch *et al.*, 1966) are not characteristic of this tumour in mares (Stabenfeldt *et al.*, 1979). However, the tumour from the mare that had higher concentrations of circulating oestradiol showed a greater degree of immunostaining. It has been suggested that aromatization of testosterone to oestradiol may be low in granulosa cell tumours (Stabenfeldt *et al.*, 1979) and our results showed that very little aromatase was present.

The results from the present study showed good agreement between expression of P-450_{arom} and recent reports of steroidogenesis by isolated cell types *in vitro*. Together with our previous results showing staining for P-450_{17 α} in theca interna cells of preovulatory follicles (Rodger *et al.*, 1995), we have confirmed that the two-cell theory of steroidogenesis, in which thecal androgen is aromatized by granulosa cells (McNatty *et al.*, 1979; Hillier, 1981), applies to the mare ovary.

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