Immunolocalization of 3 β -hydroxysteroid dehydrogenase, cytochrome P450 17 α -hydroxylase/17,20-lyase and cytochrome P450 aromatase in the equine corpus luteum of dioestrus and early pregnancy

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The onset of equine chorionic gonadotrophin (eCG) secretion in pregnant mares is associated with an increase in luteal androgen and oestrogen production. The luteal cell type(s) responsible for the increased production of androgens and oestrogens has not been identified in the equine corpus luteum. In this study, we examined the pattern of expression of 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17 α -hydroxylase/17,20-lyase (P450_{17 α}) and cytochrome P450 aromatase (P450_{arom}) by immunohistochemistry in equine luteal tissue collected during dioestrus (days 7–10; $n = 4$) and early pregnancy, before (days 29–35; $n = 4$) and after (days 39–45; $n = 4$) the onset of endogenous eCG secretion. All luteal cells expressed 3 β -HSD, P450_{17 α} and P450_{arom}. The distribution of 3 β -HSD, P450_{17 α} and P450_{arom} did not differ with stage of the reproductive cycle. The intensity of immunohistochemical staining for 3 β -HSD did not appear to differ with reproductive stage. In contrast, the intensity of immunostaining for P450_{17 α} increased after the onset of eCG secretion. The intensity of immunostaining for P450_{arom} increased during pregnancy before the onset of eCG secretion and diminished after the onset of eCG secretion to the intensity seen in dioestrous corpora lutea. This finding suggests that androgen and oestrogen production is not compartmentalized within the equine corpus luteum. Both large and small luteal cells express the steroidogenic enzymes necessary for oestrogen production, and the intensity of immunostaining for P450_{17 α} and P450_{arom} appears to be stage-specific.

Introduction

Around days 35–40 of equine pregnancy, maternal plasma androgen and oestrogen concentrations increase two to three-fold in association with the onset of eCG secretion (Nett *et al.*, 1973, 1975; Terqui and Palmer, 1979; Silberzahn *et al.*, 1984; Daels *et al.*, 1990, 1991a, 1996; Daels and Albrecht, 1995). It has been demonstrated both *in vivo* and *in vitro* that the increase in circulating androgen and oestrogen concentrations is the result of an increase in luteal steroidogenesis (Daels *et al.*, 1990, 1991a, b, 1995, 1996; Stabenfeldt *et al.*, 1991; Daels and Albrecht, 1995). In addition, administration of increasing amounts of eCG to pregnant mares on days 26–28 induces increases in plasma concentrations of androstenedione and oestrogen similar to those seen at the onset of endogenous eCG secretion (Daels and Albrecht, 1995). Similarly, luteal cells collected from pregnant mares after the onset of eCG secretion (days 40–44) secrete more oestrogen *in vitro* than do luteal cells obtained during dioestrus (days 7–10) or pregnancy before the onset of eCG secretion (days 29–35; Daels *et al.*, 1995). These

observations support the belief that the equine corpus luteum produces oestrogen regardless of reproductive stage, and that eCG regulates luteal steroidogenesis during pregnancy.

Secretion of oestrogen by the corpus luteum is not unique to horses. Corpora lutea from humans (Richardson and Masson, 1981), non-human primates (Fitz *et al.*, 1982), goats (Band *et al.*, 1987), pigs (Gregoraszczuk, 1991), ferrets (Mead and Swannack, 1980), skunks (Ravindra *et al.*, 1984), and rats (Elbaum and Keyes, 1976) secrete oestrogen. In most species examined, oestrogen is produced via the Δ^4 steroidogenic pathway which includes the enzymes 3 β -HSD (pregnenolone to progesterone), P450_{17 α} (progesterone to androgen) and P450_{arom} (androgen to oestrogen). In horses, mRNA expression (Albrecht *et al.*, 1997), enzyme activity (Al-Timimi *et al.*, 1989) and steroid secretion (Channing, 1969; Channing and Grieves, 1969; Younglai, 1971; Mahajan and Samuels, 1974; Squires *et al.*, 1978; Kelly *et al.*, 1988; Watson and Sertich, 1990; Broadley *et al.*, 1994; Daels *et al.*, 1995) have been demonstrated for these enzymes in luteal tissue, suggesting that the Δ^4 pathway is the predominant mechanism of luteal oestrogen production in mares (Albrecht *et al.*, 1997).

The corpus luteum of most species, including horses, is composed of non-luteal and luteal cells (Fitz *et al.*, 1982; Hoyer

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et al., 1988; Kelly *et al.*, 1988; Farin *et al.*, 1989). Luteal cells possess steroidogenic capacity and are designated as large or small on the basis of cellular diameter. Regulation and steroidogenic capacity differ between large and small luteal cells. In cows and sheep, basal progesterone production is greatest in large luteal cells and gonadotrophin-induced progesterone production is greatest in small luteal cells (Wiltbank, 1994). The relative contributions of large and small cells to androgen and oestrogen production is species-specific. In rhesus macaques and rats, both luteal cell types produce androgen and oestrogen, with large cells producing more androgen and oestrogen than small cells (Hild-Petito *et al.*, 1989; Smith *et al.*, 1989; Sanders *et al.*, 1996). In contrast, androgen and oestrogen production appear to be compartmentalized within the human corpus luteum; androgens are produced by the small cells and oestrogens are produced by the large cells (Fisch *et al.*, 1989). The contribution of large and small luteal cells to androgen and oestrogen production by the equine corpus luteum is unknown and limited information is available regarding the cell-specific distribution of the Δ^4 pathway enzymes within the equine corpus luteum. The presence of 3β -HSD in dispersed luteal cells obtained from dioestrus has been demonstrated histochemically (Kelly *et al.*, 1988; Watson and Sertich, 1990; Broadley *et al.*, 1994). Cytochrome P450_{arom} has been immunolocalized in luteal cells of the equine dioestrous corpus luteum (Eisenhauer *et al.*, 1994; Almadhidi *et al.*, 1995; Watson and Thomson, 1997). The effect of pregnancy and eCG on the pattern of expression of 3β -HSD, P450_{17 α} and P450_{arom} have not been determined in the equine corpus luteum.

The objective of this study was to determine the cell- and stage-specific patterns of expression of 3β -HSD, P450_{17 α} and P450_{arom} within the equine corpus luteum. The expression of these enzymes was analysed using immunohistochemical techniques in equine corpora lutea collected during dioestrus and early pregnancy, before and during the period of increased luteal oestrogen secretion associated with the onset of eCG secretion.

Materials and Methods

Animals

Light breed mares with no known reproductive pathology were used in this study. Reproductive tracts of the mares were monitored daily during oestrus and every other day from ovulation until collection of corpora lutea. Examinations were performed using palpation and ultrasonography (Aloka 210, 5 MHz linear transducer, Corometrics Medical Systems Inc., North Wallingford, CT) via the rectum. The day of ovulation was designated as day 0 of dioestrus and pregnancy. Mares used for collection of corpora lutea during pregnancy were mated by artificial insemination. Animals were maintained on grass pasture with water and trace minerals available *ad libitum*.

Sample collection

Ovaries containing the primary corpus luteum were obtained from mares in dioestrus (days 7–10; $n = 4$) and early

pregnancy, before (days 29–35; $n = 4$) and after the onset of eCG secretion (days 39–45; $n = 4$) by hemiovariectomy or after slaughter as described by Daels *et al.* (1995). After dissection from the ovary, pieces of corpus luteum were immersion-fixed in Bouin's solution and embedded in paraffin wax. Control tissues, equine testis, preovulatory follicle wall and lung, were processed in the same manner as the luteal tissue.

Antibodies

Antisera for 3β -HSD, P450_{17 α} and P450_{arom} were generously provided by V. Luu-The (Centre Hospitalier de l'Université Laval, Québec, Canada), A. H. Payne (University of Michigan, Ann Arbor, MI) and Y. Osawa (Medical Foundation of Buffalo, Buffalo, NY), respectively. The 3β -HSD antisera (lot 7545) was raised in rabbits against human placental 3β -HSD (Luu-The *et al.*, 1990). The P450_{17 α} antisera (lot MB) was raised in rabbits against pig testicular P450_{17 α} (Hales *et al.*, 1987). The P450_{arom} antisera (lot R-10-2) was raised in rabbits against human placental P450_{arom} (Kitawaki *et al.*, 1989).

Immunohistochemistry

An indirect immunoperoxidase staining method using the streptavidin/biotin technique was performed using a commercial kit (Zymed Laboratories, South San Francisco, CA). Immunolocalization was performed on tissue sections 4–6 μ m thick mounted on slides coated with poly-L-lysine (Sigma, St Louis, MO). Sections were deparaffinized by incubation at 70°C for 30 min, followed by three, 5 min changes of xylenes. Endogenous peroxidase activity was quenched by incubation of the sections in methanol containing 0.5% H₂O₂ for 10 min at room temperature followed by three, 5 min rinses in 0.01 mol PBS l⁻¹. The tissues were then incubated with 10% normal goat serum for 10 min at room temperature, and then incubated with primary antibody for 18 h at 4°C. Antisera were diluted in PBS; 1:2000 for 3β -HSD and P450_{arom}, and 1:1000 for P450_{17 α} . Subsequent to three, 5 min rinses in PBS, the sections were incubated with a goat anti-rabbit biotinylated secondary antibody at room temperature for 5 min, and then given three, 5 min rinses in PBS. The sections were incubated with streptavidin–peroxidase conjugate for 5 min at room temperature, and then given three, 5 min rinses in PBS. The antigen–antibody complex was visualized by incubating the sections with the chromogen substrate, 0.05% (v/v) 3,3'-diaminobenzidine (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) and 0.01% H₂O₂ for 15 min at room temperature. Tissues were counterstained with Gil's number 2 Haematoxylin (Fisher Scientific, Springfield, NJ) and mounted with Permount (Fisher Scientific). Controls included substitution of the primary antibody with PBS or non-immune rabbit sera at the same dilution as the primary antibody. Tissue controls included equine preovulatory follicle wall, testis and lung.

The pattern and intensity of staining for each enzyme were determined by visual inspection. Sections of luteal tissue obtained at all three reproductive stages and stained in the same batch were compared to determine differences in staining pattern and intensity among stages.

Results

3 β -Hydroxysteroid dehydrogenase

Immunoreactivity for 3 β -HSD was observed in the cytoplasm of all luteal cells (Fig. 1b–d) for the three reproductive stages examined. The intensity of the immunohistochemical signal did not appear to differ among reproductive stages. Immunoreactivity for 3 β -HSD was also present in the granulosa cells of the preovulatory follicle (Fig. 1a) and in the Leydig cells of the testis (Fig. 2b). Weak immunoreactivity was observed in the theca interna layer of the follicle wall (Fig. 1a). No immunoreactivity was observed within the connective tissue trabeculae of the corpus luteum (Fig. 1b–d), the seminiferous tubules of the testis (Fig. 2b), or the lung parenchyma (Fig. 2a).

Cytochrome P450 17 α -hydroxylase/17,20-lyase

Immunoreactivity for P450_{17 α} was observed in the cytoplasm of all luteal cells for the three reproductive stages examined (Fig. 1f–h). The intensity of the immunohistochemical signal appeared similar in corpora lutea obtained during dioestrus (days 7–10) and early pregnancy before the onset of eCG secretion (days 29–35). In corpora lutea obtained after the onset of eCG secretion (days 39–45) the intensity of the immunohistochemical signal appeared stronger than at the other two stages examined. The theca interna of the preovulatory follicle (Fig. 1e) and the Leydig cells of the equine testis (Fig. 2d) stained positively for P450_{17 α} . Weak immunoreactivity was observed in the granulosa cells of the preovulatory follicle (Fig. 1e). No immunoreactivity was observed within the connective tissue trabeculae of the corpus luteum (Fig. 1f–h), the seminiferous tubules of the testis (Fig. 2d), or the lung parenchyma (Fig. 2c).

Cytochrome P450 aromatase

Immunoreactivity for P450_{arom} was observed in the cytoplasm of all luteal cells for the three reproductive stages examined (Fig. 1j–l). The intensity of the immunohistochemical signal appeared strongest in corpora lutea collected during early pregnancy before the onset of eCG secretion (days 29–35). Immunoreactivity was similar in luteal tissue from dioestrus (days 7–10) and early pregnancy after the onset of eCG secretion (days 39–45), although the signal was weaker than in corpora lutea collected during early pregnancy before the onset of eCG secretion (days 29–35). Occasional individual luteal cells stained more intensely for P450_{arom} at each reproductive stage (Fig. 1j–l). The granulosa cells of the preovulatory follicle (Fig. 1i) and the Leydig cells of the testis (Fig. 2f) stained positively for P450_{arom}. Weak immunoreactivity was observed in the theca interna layer of the preovulatory follicle (Fig. 1i). No immunoreactivity was observed within the connective tissue trabeculae of the corpus luteum (Fig. 1j–l), the seminiferous tubules of the testis (Fig. 2f), or the lung parenchyma (Fig. 2e).

Replacement of the primary antibody with non-immune rabbit sera or buffer did not show immunoreactivity in any tissue (data not shown).

Discussion

The results of this study demonstrate the presence of 3 β -HSD, P450_{17 α} and P450_{arom} enzymes in equine luteal tissue. All luteal cells stained positively for 3 β -HSD, P450_{17 α} and P450_{arom}, suggesting that the steroidogenic enzyme cascade for oestrogen production is not compartmentalized within the equine corpus luteum during dioestrus or early pregnancy, before or after the onset of eCG secretion.

All luteal cells within the equine corpus luteum were positive for 3 β -HSD regardless of reproductive stage examined. These results agree with the results of immunohistochemical studies in guinea pigs (Dupont *et al.*, 1990), rats (Juneau *et al.*, 1993) and humans (Sasano *et al.*, 1990; Dupont *et al.*, 1992), and with previous histochemical analyses of dissociated equine luteal cells (Kelly *et al.*, 1988; Broadley *et al.*, 1994). Similarly, equine luteal 3 β -HSD activity has been confirmed *in vitro* (Channing, 1969; Channing and Grieves, 1969; Younglai, 1971; Squires *et al.*, 1978; Kelly *et al.*, 1988; Al-Timimi *et al.*, 1989b; Amri *et al.*, 1993a; Broadley *et al.*, 1994; Daels *et al.*, 1995). No difference was observed in staining intensity between large and small luteal cells, suggesting equal expression of 3 β -HSD by each cell type. How the expression of 3 β -HSD relates to the relative contribution of large and small luteal cells to total progesterone production by the equine corpus luteum remains unknown. In the bovine and ovine corpus luteum, basal progesterone production by large cells is greater than by small cells and, under the influence of LH, small cell progesterone production is stimulated more than large cell progesterone production (Bourdage *et al.*, 1984; Alila *et al.*, 1988; Hansel *et al.*, 1991). The intensity of 3 β -HSD immunostaining did not appear to differ with reproductive stage and this is in agreement with previous studies from our laboratory (Daels *et al.*, 1995; Albrecht *et al.*, 1997). In these studies, mixed populations of dissociated equine luteal cells produced progesterone at the same rate (Daels *et al.*, 1995) and steady-state amounts of luteal mRNA encoding 3 β -HSD did not differ (Albrecht *et al.*, 1997) among the reproductive stages examined. The results of the present study suggest that pregnancy and eCG do not affect the pattern or amount of 3 β -HSD expression within the equine corpus luteum.

The presence of P450_{17 α} and P450_{arom} as demonstrated by immunohistochemical staining, is in agreement with a majority of previous studies of the equine corpus luteum. Dioestrous corpora lutea express P450_{arom} in all luteal cells (Eisenhauer *et al.*, 1994; Almadhidi *et al.*, 1995). The equine corpus luteum produces significant quantities of androgen and oestrogen *in vivo* (Daels *et al.*, 1990, 1991b, 1996; Stabenfeldt *et al.*, 1991; Daels and Albrecht, 1995). Similarly, luteal microsomes and dissociated luteal cells produce androgens and oestrogens *in vitro* (Al-Timimi *et al.*, 1989; Amri *et al.*, 1993a, b; Daels *et al.*, 1995). The immunostaining for P450_{17 α} and P450_{arom} enzymes in both large and small luteal cells suggests that all luteal cells of the equine corpus luteum possess the enzymes necessary for androgen and oestrogen production. The results of the present study are in contrast to studies of humans, in which expression of P450_{17 α} and P450_{arom} is compartmentalized in a cell-specific manner within the corpus luteum (Sasano *et al.*, 1989; Naganuma *et al.*, 1990; Inkster and Brodie, 1991; Tamura *et al.*, 1992). Cytochrome P450_{17 α} immunoreactivity is limited

Fig. 1. Immunolocalization of steroidogenic enzymes in sections (4–6 μm) of paraffin wax-embedded equine preovulatory follicle (a, e, i) and corpora lutea from dioestrus (b, f, j) and early pregnancy before (c, g, k) and after (d, h, l) the onset of equine chorionic gonadotrophin secretion. Tissues were stained for 3̢-hydroxysteroid dehydrogenase (a–d), cytochrome P450 17̡-hydroxylase/17,20-lyase (e–h) and cytochrome P450 aromatase (i–l) using an indirect immunoperoxidase method. Positive staining is indicated by brown deposits. All micrographs were taken at the same magnification and the scale bar represents 126 μm .

Fig. 2. Immunolocalization of steroidogenic enzymes in equine lung (a, c, e) and testis (b, d, f). Tissues were stained for 3 β -hydroxysteroid dehydrogenase (a, b), cytochrome P450 17 α -hydroxylase/17,20-lyase (c, d) and cytochrome P450 aromatase (e, f) using an indirect immunoperoxidase method. Positive staining is indicated by brown deposits. All micrographs were taken at the same magnification and the scale bar represents 126 μ m.

to the small, theca-lutein cells, and P450_{arom} immunoreactivity is limited to the large, granulosa-lutein cells of the human corpus luteum (Sasano *et al.*, 1989; Naganuma *et al.*, 1990; Inkster and Brodie, 1991; Tamura *et al.*, 1992). Thus, the two-cell system of steroidogenesis, as described in the human corpus luteum and the antral follicle of most species, including the horse, appears to be absent from the equine corpus luteum. The difference in pattern of immunoreactivity may be due to the differences in luteal development between horses and humans. Studies on the development of the equine corpus luteum suggest that the luteal parenchyma is derived from the granulosa cells (Harrison, 1946; Levine *et al.*, 1979). In contrast, the parenchyma of the human corpus luteum is derived from both theca and granulosa cells of the follicle (Ohara *et al.*, 1987; Sasano *et al.*, 1989). If the equine corpus luteum is derived solely from granulosa cells, it would suggest that the process of luteinization activates expression of P450_{17 α} . As seen in the human corpus luteum (Tamura *et al.*, 1992), the cell-specific patterns of P450_{17 α} and P450_{arom} expression did not change with reproductive stage, suggesting that once luteinization occurs, the population of cells involved in androgen and oestrogen production does not change within the equine corpus luteum.

Prominent staining for P450_{arom} was observed in all luteal cells regardless of reproductive stage. Throughout the luteal tissue, occasional luteal cells exhibited darker staining but the incidence of these cells was not stage-specific. This observation is in contrast to recent observations by Watson and Thompson (1997) who noted only pale staining for P450_{arom} in most luteal cells and an increase in darkly staining cells after day 40 of gestation. Differences observed in the two studies may be due to methodology.

The intensity of P450_{17 α} and P450_{arom} immunostaining differed with reproductive stage. Immunostaining for P450_{17 α} appears to parallel expression of mRNA encoding P450_{17 α} within the equine corpus luteum (Albrecht *et al.*, 1997). Steady-state amounts of mRNA encoding P450_{17 α} are not different in corpora lutea collected during dioestrus or early pregnancy before the onset of eCG secretion. After the onset of eCG secretion, steady-state amounts of P450_{17 α} are significantly increased compared with amounts in corpora lutea at the previous reproductive stages. The intensity of P450_{arom} immunostaining appeared highest in corpora lutea obtained during pregnancy before the onset of eCG secretion. Corpora lutea from dioestrus or early pregnancy after the onset of eCG secretion stained similarly, but less intensely, than corpora lutea

from early pregnancy before the onset of eCG secretion. These data are similar to results obtained from northern blot analysis of equine corpora lutea (Albrecht *et al.*, 1997). Amounts of cytochrome mRNA encoding P450_{arom} are highest in corpora lutea obtained during early pregnancy before the onset of eCG secretion and are lowest during pregnancy after the onset of eCG secretion when compared with expression during dioestrus. The parallelism between mRNA expression and intensity of immunostaining for P450_{arom} and P450_{17 α} suggest that transcription and translation are closely coupled for these enzymes within the equine corpus luteum.

These results support the hypothesis that before the onset of eCG secretion luteal oestrogen production is limited by the expression of P450_{17 α} , and that the availability of aromatizable androgen substrate is the primary regulatory mechanism of luteal oestrogen production. During gestation in rats, P450_{arom} immunoreactivity and protein increase from day 7 to days 15–19 in the corpus luteum (Hickey *et al.*, 1988; Juneau *et al.*, 1993) and are paralleled by increases in mRNA encoding P450_{arom} (Hickey *et al.*, 1988). Although the content of aromatase is maximal in the rat corpus luteum, the production of oestradiol is submaximal (Hickey *et al.*, 1988), further suggesting that availability of androgens is a primary regulator within the corpus luteum. The temporal association between changes in the intensity of immunostaining for P450_{17 α} and P450_{arom} and the onset of eCG secretion in horses suggests that eCG differentially regulates the expression of both enzymes, and that androgen and oestrogen production is regulated primarily by the expression of P450_{17 α} .

The immunoreactive patterns of P450_{17 α} and P450_{arom} in the equine preovulatory follicle confirm the compartmentalization of androgen and oestrogen production, with P450_{17 α} expressed primarily in the theca interna layer, and P450_{arom} expressed primarily in the granulosa layer. Light immunoreactivity in the granulosa layer and the theca interna layers for P450_{17 α} and P450_{arom}, respectively, were noted. Isolated granulosa cells from the preovulatory follicle have the ability to produce limited basal quantities of androstenedione and oestradiol *in vitro* (Sirois *et al.*, 1991). When testosterone is added to the culture medium, oestradiol production by isolated granulosa cells is significantly increased (Sirois *et al.*, 1991). Isolated theca interna from equine preovulatory follicles produce predominantly androstenedione (Sirois *et al.*, 1991). Therefore, the results of the present study provide additional immunohistochemical evidence for the two-cell theory of steroidogenesis within equine preovulatory follicles.

In conclusion, 3 β -HSD, P450_{17 α} and P450_{arom} have been demonstrated to be expressed by the equine corpus luteum of dioestrus and early pregnancy. These results provide evidence for the presence of the Δ^4 steroidogenic pathway and the absence of a two-cell mechanism of androgen and oestrogen production within the equine corpus luteum. The temporal association between the onset of eCG secretion and the changes in immunoreactive intensity for P450_{17 α} and P450_{arom} support the hypotheses that P450_{17 α} is the primary regulatory step in luteal androgen and oestrogen production, and that eCG differentially regulates P450_{17 α} and P450_{arom} within the equine corpus luteum.

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