Evidence that a non-steroidal factor from corpus luteum of pregnant sheep inhibits aromatase activity of ovarian follicles in vitro

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Porcine and ovine follicular tissues were used to investigate, in vitro, the effect of charcoal-treated aqueous extract from ovine corpora lutea of pregnancy on aromatase activity as determined by the conversion of [¹¹C]testosterone to oestradiol by follicular walls and measurement of [¹¹C]H₂O release. Extract (500 μg protein) prepared from corpora lutea of day 112 of pregnancy but not extract (500 μg) prepared from ovine fetal cotyledonary tissue obtained at a similar time significantly decreased (P < 0.02) aromatase activity of pig follicles in the absence of FSH. These results demonstrate that a non-steroidal factor in the corpora lutea of late pregnancy directly inhibits aromatase activity. When the effects of different doses (300, 600 or 1200 μg) of luteal extract from corpora lutea of day 100 of pregnancy on aromatase activity of pig follicles were studied, the dose by treatment (presence or absence of FSH) interaction was not significant. Luteal extract dose at 300 μg did not affect aromatase activity but a significant decrease in activity occurred at 600 μg of luteal extract (600 versus 300 μg, P < 0.02). There was no further significant increase in the inhibitory effect with 1200 μg luteal extract. When the effects of 600 μg luteal extract from corpora lutea of days 15, 75 or 100 of pregnancy on aromatase activity of pig follicles were studied, a significant (P < 0.05) stage of pregnancy effect was detected, but the stage of pregnancy by treatment (presence or absence of FSH) interaction was not significant. No effect was noted with day 15 or day 75 luteal extract. In contrast, aromatase activity in the presence of day 100 luteal extract was significantly reduced compared with that of control (P < 0.01) and day 15 luteal extract (P < 0.05). A significant (P < 0.05) stage of pregnancy effect was also observed on aromatase activity of sheep follicles. Aromatase activity of sheep follicles was significantly reduced in the presence of day 100 luteal extract compared with that of control (P < 0.05) and day 15 luteal extract (P < 0.02). These data suggest that the stimulus triggering the synthesis of the aromatase inhibitor appears after mid-pregnancy. The aromatase-inhibiting activity was lost from luteal extract of corpora lutea of day 100 of pregnancy after treatment with proteolytic enzymes, demonstrating the protic nature of the aromatase inhibitor. These experiments provide evidence for the existence in ovine corpora lutea of late pregnancy of a non-steroidal factor that reduces follicular aromatase activity. We propose the term aromatase-inhibiting factor or AlF to describe this activity.

Introduction

Non-atretic follicles greater than 2 mm in diameter are present continuously in ovaries of ewes throughout the oestrous cycle (Hutchinson and Robertson, 1966; Brand and De Jong, 1973). In contrast, the growth of antral follicles decreases progressively as pregnancy advances (Williams et al., 1956; Rüsse, 1971) and the largest follicles never exceed 2 mm during their last month (Smeaton and Robertson, 1971; Rüsse, 1971; Al-Gubory and Martinet, 1986). At any time during the oestrous cycle, only 27–67% of the 1–2 mm follicles are atretic (Brand and De Jong, 1973), but most (97%) of these follicles exhibit signs of atresia during late pregnancy (Al-Gubory and Martinet, 1986).

Although it is now generally accepted that the growth of follicles beyond 2 mm in the nonpregnant ewe depends upon pituitary gonadotrophins (Dufour et al., 1979; McNeilly et al., 1986; Driancourt et al., 1987; McNeilly and Fraser, 1987; McNatty et al., 1990) particularly FSH (Picton et al., 1990a, b), little is known about the factors involved in the inhibition of
the final stages of follicular growth and function in pregnant ewes. The disturbance of antral follicular growth during pregnancy in ewes is usually ascribed to the marked increases after mid-pregnancy in peripheral progesterone concentrations (Bassett et al., 1969) that exerts a negative feedback effect on pituitary gonadotrophin secretion. As there is no change in FSH secretion as pregnancy progresses (Al-Gubory et al., in press), there are three possible candidates that could account for the inhibition of follicular growth at the ovarian level (1) the fetoplacental unit, (2) the corpus luteum or (3) an interaction between these factors. Evidence supporting a role for the fetoplacental unit has been obtained by Al-Gubory et al. (1992a), who showed that ablation of the gravid uterus at day 31 after matting resulted in the presence of large (>2 mm) healthy follicles at day 120 after mating, despite the lack of hysterectomy induced changes in FSH and LH secretion (Al-Gubory et al., in press). Evidence supporting a role for the corpus luteum is that the corpora lutea of mid-pregnancy secrete (Al-Gubory, 1987; Al-Gubory et al., 1989a,b) and contains a proteic factor (Al-Gubory et al., 1992b) that inhibits pulsatile release of LH in ewes. Whether this compound or other corpus luteum or placent factors act locally to inhibit follicular function during late pregnancy is unknown. To clarify thispoint, we examined the effects of luteal or placental tissue extracts on aromatase activity by ovarian follicles in vitro.

Materials and Methods

Preparation of tissue extracts

Corpora lutea of early (day 15), mid-(day 75), and late (days 100–120) pregnancy, and fetal cotyledons of day 112 of pregnancy were collected at the local abattoir from ewes of the Prêalpes-du-Sud breed, frozen in liquid nitrogen and stored at −20°C until extracted. Luteal and placental tissues were thawed, minced and homogenized in ice-cold saline (0.9% NaCl). The homogenate was stirred for 20 min and then centrifuged for 30 min at 20 000 g. Steroids were removed by treating the resulting supernatants with dextran-coated charcoal as described by Al-Gubory et al. (1992b). After charcoal extraction, the progesterone concentrations were 3.6, 3.0 and 3.6 ng ml−1, and the oestradiol concentrations were 0.07, 0.12 and 0.10 ng ml−1 of luteal extract from corpora lutea of days 15, 75 and 100 of pregnancy, respectively. Testosterone was not detected in luteal extracts of any stage of pregnancy (<0.2 ng ml−1). After charcoal treatment, the supernatants were centrifuged for 1 h at 105 000 g, filtered through a Millex-HV 0.45 μm filter (Millipore, Molsheim) and then sterilized by filtration through a Millex-GV 0.22 μm filter (Millipore) before use. All steps of tissue extract preparation were conducted at 4°C. Protein concentrations of the different tissue extracts were determined by the method of Lowry et al. (1951), using BSA as the standard. The amount of protein extracted per corpus luteum of early, mid- and late pregnancy was 34, 43 and 37 mg, respectively.

Assay of the aromatase inhibitory activity of tissue extracts

Large ovulatory follicles (5.0–7.5 mm in diameter) were obtained from Meishan pigs (Expts 1, 2, 3a and 4) and Prêalpes sheep (Expt 3b). Pig follicles were used in most of this study because (1) a large amount of follicular material is available in this species and (2) sheep and pig follicles behaved in the same way (Expts 3a, b). Pig follicles were obtained on the morning of day 5 after the end of the altronestogest treatment for 18 days at 20 ng day−1 (Roussel Uclaf, Romainville). Sheep follicles were obtained 30 h after the end of treatment for 14 days with fluorogestone acetate impregnated sponges (Intervet, Angers).

After careful dissection and measurement, follicular fluid was aspirated and each follicle was divided into two (Expt 1), 3 (Expt 4) and 4 (Expts 2, 3a, b) fragments. Each fragment was incubated at 37°C for 24 h in B2 Ménézo medium (600 μl per well; bioMérieux, Marcy L’Etoile) under 95%O2:5% CO2. All test substances were added at the beginning of culture. Ten hours after the beginning of culture, 500 000 d.p.m. of [3H]testosterone (Du Pont de Nemours, Les Ulis) were added. The amount of testosterone added to the follicle cultures was 20 ng per well, and the specific activity of the steroid tracer was 40–60 Ci mmol−1. The 10 h lag before adding the [3H]testosterone was selected because it is known that this is the time required for FSH to act on aromatization in pig (Westhoff et al., 1989) and sheep (M-A. Driancourt and R. C. Fry, unpublished data). Follicles. At the end of the culture, the medium was removed and stored frozen, while each follicle quarter was blotted dry and carefully weighed.

Aromatase activity was measured by the release of 3H2O after the conversion of [3H]testosterone to oestradiol as described by Gore Langion and Dorrington (1981) and validated by Thatcher et al. (1991). Aromatase activity was expressed as d.p.m. 3H2O/d.p.m. 3H2O + d.p.m. [3H]testosterone mg−1 tissue. Validation of this procedure demonstrated that (1) aromatase activity was partly maintained during culture and was 55% and 70% in sheep and pigs, respectively, between 20 and 24 h of culture of the value at 0–4 h of culture (Driancourt, 1992); (2) aromatase activity of two fragments of the same follicle is highly correlated (n = 18, r = 0.65, P < 0.001); (3) aromatase activity can be increased or decreased by treatment with FSH and follicular fluid, respectively (Driancourt, 1992; M-A. Driancourt and R. C. Fry, unpublished data).

Experimental design

Experimental 1: detection of aromatase inhibitor in luteal or placental extracts from day 112 of pregnancy. Follicles (n = 28) were dissected from two sows, and randomly allocated to four groups: 160 μg or 500 μg of luteal (n = 10 and 5, respectively) or placental (n = 8 and 5, respectively) extracts. Each follicle was divided in two parts, one part was kept as a control and the other was challenged with the test substance.

Experimental 2: dose–response relationship in the presence or absence of FSH. Fourteen follicles (mean diameter 6.7 mm) were dissected from one sow, and divided into four. Quarter 1 was kept as a control, while quarters 2, 3 and 4 were treated, respectively, with 300, 600 and 1200 μg luteal extract from day 100 of pregnancy. Eight follicles were cultured in basal conditions and six follicles were cultured in the presence of 10 ng porcine FSH (CY 17373II) which has a potency of 41 FSH NIH P1 and exhibits crossreactions with LH not exceeding 1%).
Experiment 3: effect of stage of pregnancy on the amount of inhibition. In Expt 3a, follicles (n = 21) from two sows were divided into four. Quarter 1 was kept as control, while quarters 2, 3 and 4 were treated with 600 μg luteal extracts from days 15, 75 or 100 of pregnancy. Twelve follicles were cultured in basal conditions and nine follicles in the presence of 10 ng porcine FSH.

In Expt 3b, follicles (n = 9) from seven Préalpes ewes were treated as in Expt 3a except that all of them were cultured in the presence of 2 ng ovine FSH ml⁻¹ (CY 1746, which has a potency of 50 FSH NIH S₄, and exhibits crossreactions with LH and TSH not exceeding 1%).

Experiment 4: effect of proteases on aromatase inhibitory activity. Ten follicles (mean diameter 6.5 mm) from one sow were divided into three parts. Part 1 was kept as a control while parts 2 and 3 were treated with 600 μg luteal extract or with 600 μg protease-treated luteal extract, from days 100–120 of pregnancy. Protease treatment involved incubation of charcoal-treated extract at 37°C for 3 h with proteinase K (200 μg ml⁻¹) and then for 3 h with pronase (200 μg ml⁻¹).

Statistical analysis

Data were analysed after transformation (arc sin square root) by paired t tests (Expt 1), by one-way ANOVA for repeated measurements (Expt 3b and 4) or by two-way ANOVA for repeated measurements (Expts 2 and 3a) conducted on a paired basis.

Results

Experiment 1

A significant (P < 0.02) decrease in aromatase activity was found in the half of the follicle treated with 500 μg luteal extract (69% of the control value), but not in that treated with 100 μg (102% of the control half) (Fig. 1). Aromatase activity was unaffected by both concentrations of placental extracts (160 or 500 μg) and was 110 and 97% of the values of the control halves, respectively.

Experiment 2

When the effects of different doses of luteal extract from corpora lutea of day 100 on aromatase activity of pig follicles were studied, the dose by treatment (presence or absence of FSH) interaction was not significant. Data for the effects of luteal extract in the presence and absence of FSH were therefore pooled. No effect on aromatase activity was noted with day 15 or day 75 luteal extract. In contrast, aromatase activity was significantly lower in the presence of day 100 luteal extract than with control (P < 0.01) and day 15 luteal extract (P < 0.05) (Fig. 3).

A significant (P < 0.05) stage of pregnancy effect was observed on aromatase activity of sheep follicles (Expt 3b). Aromatase activity in the presence of day 100 luteal extract was significantly lower (Fig. 4) than in controls (P < 0.05) and with day 15 luteal extract (P < 0.02).

Experiment 4

Aromatase activity of pig follicles was unaffected by protease-treated luteal extract from corpora lutea of days 100–120 of pregnancy, but was significantly different (P < 0.05) from that observed in the presence of crude luteal extract (Fig. 5).

Discussion

The results of the present experiments provide evidence for the existence in ovine corpora lutea of late pregnancy of a non-steroidal factor that reduces the aromatase activity of antral follicles. The aromatase inhibitor appears to be primarily of luteal origin, as tissue extract from corpora lutea of day 112.
of pregnancy affected aromatase activity, whereas that from fetal cotyledons did not. Aromatase activity of porcine or ovine antral follicles was unaffected after treatment with luteal extracts from corpora lutea of early (day 15) or mid- (day 75) pregnancy, but reduced by extracts from corpora lutea of day 100 of pregnancy. This finding suggests that production of the aromatase inhibitor increases with the advance of pregnancy and that the corpora lutea produce larger quantities of this factor during the last third, when follicular growth was limited to follicles with a diameter of < 2 mm and most of the follicles > 1 mm were atretic (Al-Gubory and Martinet, 1986). The inhibition of aromatase activity by luteal extract was destroyed after treatment with proteolytic enzymes. Hence, the aromatase inhibitor appears to be a protein. The degree of the inhibition by luteal extract from late corpora lutea of pregnancy on follicular aromatase activity in the presence or absence of FSH was similar. These results therefore suggest that the aromatase inhibitor described in the present study has biological properties different from those of follicular secretory proteins identified in a variety of mammalian species, referred to as FSH-binding inhibitor (Darga and Reichert, 1978; Sluss and Reichert, 1984) and follicle regulatory protein (dizerega et al., 1982; Kling et al., 1984), that inhibit follicular response to gonadotrophins, and from those present in the wallaby quiescent corpora lutea that suppress FSH-stimulated aromatase activity (Shaw and Brinklow, 1992). As relaxin stimulates aromatase activity of human endometrial cells (Tseng et al., 1987) and pig follicles (M-A. Driancourt and P. Guet, unpublished data), the aromatase inhibitor activity is distinct from that of relaxin.

Ovarian follicular populations and their relationships to the corpus luteum have been described in sheep. Observations of visible follicles showed that the corpora lutea ovary contains more follicles > 4 mm than does the non-corpora lutea ovary on days 7 and 35, but by day 75 ovaries were devoid of follicles > 4 mm, and the number of follicles < 4 mm was similar in both ovaries on days 75 and 120 of pregnancy (Rexroad and Casida, 1975). When the total number of follicles > 0.15 mm was determined on day 140 of pregnancy (Al-Gubory and Martinet, 1987), it was found that the diameter of the largest non-atretic and atretic follicles were never larger than 1 and 1.5 mm, respectively, and was similar in the corpora lutea ovary and non-corpora lutea ovary. Follicular growth stops at follicular size of 2 mm in hypophysectomized ewes (Dufour et al., 1979; Driancourt et al., 1987; McNatty et al., 1990). These authors suggested that follicular growth beyond 2 mm is under the direct control of pituitary gonadotrophins, FSH and LH. Picton et al. (1990a, b) demonstrated that, in ewes the growth of follicles beyond 2 mm depends upon FSH. The marked inhibition of antral follicular growth appearing after mid-pregnancy in ewes (Räsä, 1971) could thus be the result of the decrease of pituitary gonadotrophin secretion, in particular FSH. However, we showed (Al-Gubory et al., in press) that concentrations of LH and FSH in plasma remain fairly constant during pregnancy. Another possibility is that local factors either inhibiting cell replication or follicle maturation are involved. Although the existence in the late pregnant corpora lutea of a factor modulating cell replication has not yet been documented, production of an aromatase inhibitor could

![Fig. 2. The effects of treatment with different doses of ovine charcoal-treated aqueous luteal extracts (■) control; (□) 300 μg extract; (□) 600 μg extract; (□) 1200 μg extract) from day 100 of pregnancy on aromatase activity of pig follicle quarters cultured in the absence and presence of 10 ng porcine FSH. Treatment effects are expressed as a percentage of aromatase activity of the untreated control quarter. Note that the baseline begins at 50% of control. *Significant difference (P < 0.02) between 300 and 600 μg extract.](image1)

![Fig. 3. The effects of ovine charcoal-treated aqueous luteal extracts (600 μg) from corpora lutea of (□) early (day 15) (□) mid- (day 75) or (□) late (day 100) pregnancy on aromatase activity of pig follicle quarters cultured in the absence and presence of 10 ng porcine FSH; (■) control. Treatment effects are expressed as a percentage of aromatase activity of the untreated control quarter. Note that the baseline begins at 50% of control. *Significant difference (P < 0.01) compared with control value.](image2)
be involved in the absence of follicular growth over 2 mm in diameter during late pregnancy. Aromatase is a key enzyme in follicular development which starts to increase when follicles reach 2 mm in diameter (Tsonis et al., 1984). Furthermore, high levels of aromatase activity are found only in large healthy follicles, whereas as soon as atresia occurs, aromatase activity falls (Tsonis et al., 1984). Hence, it is possible that factors impairing the appearance or development of aromatase activity could impair growth of large follicles. One consequence of a blockade of aromatase activity could be the accumulation of follicular androgens. Studies in hypophysectomized rats indicated that androgens are antagonistic to follicular development induced by pituitary gonadotrophins (Payne et al., 1956; Payne and Runser, 1958; Louvet et al., 1975; Zeleznik et al., 1979). Furthermore, circumstantial evidence suggests that androgens also directly antagonized antral follicular maturation in ewes. The number of follicles >3 mm increased in ewes actively immunized against androstenedione (Scaramuzzi et al., 1980) or testosterone (Scaramuzzi et al., 1981), although FSH concentration was unaffected or decreased in androstenedione or testosterone immunized ewes (Martensz and Scaramuzzi, 1979; Martensz et al., 1979; Philipon et al., 1989). The aromatase inhibitor identified in the ovine corpora lutea (present study) could therefore be directly involved in the inhibition of antral follicular growth after mid-pregnancy by increasing accumulation of follicular androgens. Since follicular growth is blocked in the ovary bearing the corpora lutea and the contralateral one at the same stage of late pregnancy (Rexroad and Casida, 1975; Al-Gubory and Martinet, 1987), it has to be assumed that the aromatase inhibitor is secreted outside the corpora lutea ovary and also acts as an endocrine regulator.

On day 120 after mating, both the corpora lutea ovary and non-corpora lutea ovary of ewes from which the gravid uteri were removed on day 31 after mating contain healthy follicles >2 mm, while those of pregnant ewes do not (Al-Gubory et al., 1992a). These findings, together with the fact that concentrations of FSH and LH in plasma do not change after hysterectomy performed on day 31 after mating, and are similar in pregnant and hysterectomized ewes between days 20 and 120 after mating (Al-Gubory et al., in press), suggest that the conceptus or the gravid uterus secretes a substance that interferes directly with follicle growth, thereby preventing the development of large healthy follicles during late pregnancy. In the present study, the finding that luteal extract from corpora lutea of late pregnancy, but not that from corpora lutea of early or mid-pregnancy, reduces follicular aromatase activity suggests that some factor(s) present and associated with pregnancy, probably originating from the conceptus or the uterus, is necessary for production of the aromatase inhibitor by the luteal cells after mid-pregnancy. The specific messenger from the conceptus or the gravid uterus required for the production of the aromatase inhibitor remains to be identified.

In conclusion, the present report provides the first experimental evidence for the existence in the corpora lutea of pregnancy of a protease-sensitive factor that inhibits follicular aromatase activity. The term aromatase inhibiting factor or AIF is proposed to describe this activity.
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