The role of pregnenolone-metabolizing enzymes in the regulation of oestradiol biosynthesis during development of the first wave dominant follicle in the cow

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Abstract

During the luteal phase in the cow, a first-wave dominant follicle grows to reach ovulatory size, but then ceases to grow, becomes no longer dominant and enters a phase of slow regression. During this growth transition, the concentration of oestradiol has been shown to decrease in follicular fluid. The objective of this study was to determine if follicular fluid oestradiol concentrations are regulated by the activity of three major steroidogenic enzymes, namely P450-aromatase (P450-arom), 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) and 17α-hydroxylase cytochrome P450 enzyme (P450-17α) measured in granulosa and theca cells isolated from individual first-wave dominant follicles. Follicle growth and state of dominance was assessed by ultrasonography and follicles were classified as growing-dominant (GD, n=6), non-growing-dominant (NGD, n=8) or non-growing-non-dominant (NGND, n=6). Mean follicular fluid concentrations of oestradiol were higher in GD than in NGD or NGND follicles (511 ± 98 versus 136 ± 16 and 20 ± 11 nmol/l respectively). Oestradiol was not correlated with P450-arom in any of the three groups. In GD follicles, oestradiol was positively correlated with pregnenolone concentration but neither was correlated with granulosa or theca 3β-HSD activity or with theca P450-17α activity. In NGD follicles, oestradiol was negatively correlated with theca 3β-HSD activity and pregnenolone was negatively correlated with granulosa 3β-HSD activity. These studies demonstrated that pregnenolone supply is the principal regulating factor of oestradiol output during follicle dominance and during the loss of dominance but that the levels of P450-17α and 3β-HSD activity become rate-limiting when the follicle is no longer dominant.

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Introduction

Ovarian ultrasonography in the cow has shown that there are two or three waves of follicular growth during the oestrous cycle (Pierson & Ginther 1984, Savio et al. 1988, Sirois & Fortune 1988). Each wave consists of a cohort of follicles from which a single large follicle emerges; it is the large follicle of the last wave which ovulates. These large follicles are said to be dominant because they continue to grow while the other follicles of the wave regress. The dominant follicle emerging from the first wave grows to reach ovulatory size by days 5–8 (day 0=oestrus) and then enters a static growth phase where it remains temporarily dominant before entering a slowly regressing phase (Savio et al. 1988, Sirois & Fortune 1988, Ginther et al. 1989). During growth, this dominant follicle is oestrogenic. Receptors for luteinizing hormone (LH) appear in granulosa cells (Ireland & Roche 1983), elevated oestradiol concentrations are found in follicular fluid (Badinga et al. 1992, Guilbault et al. 1993) and plasma oestradiol concentrations increase (Dieleman et al. 1986, Badinga et al. 1992, Carrière et al. 1995). However, this follicle fails to ovulate and loses the capacity to secrete oestradiol (Ireland & Roche 1983, Badinga et al. 1992, Guilbault et al. 1993).

Steroid hormones, produced by the antral follicle, act in autocrine, paracrine or endocrine fashions to complete the maturation process of the oocyte and to generate the signal for ovulation. Steroid hormone production follows pathways that depend upon the availability of substrate and the activities of specific steroidogenic enzymes. Follicular steroidogenesis is stimulated by follicle-stimulating hormone (FSH) and LH which, following receptor interaction, initiate a sequence of events leading to the generation of cholesterol for conversion to pregnenolone in...
mitochondria. Pregnenolone is converted to progesterone, androgens and oestrogens by enzymes residing in the endoplasmic reticulum. In theca cells of large bovine follicles the favoured steroidogenic pathway involves conversion of pregnenolone to dehydroepiandrosterone (DHEA) by the 17α-hydroxylase C-17,20 lyase cytochrome P450 enzyme (P450-17α) and conversion of DHEA to androstenedione by the enzyme 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD) (Lacroix et al. 1974, Fortune 1986). Bovine granulosa cells convert androgens supplied by the theca cells to oestradiol by the P450-aromatase enzyme (P450-arom) (Lacroix et al. 1974, McNatty et al. 1984, Cooke & Carrière 1991). In the cow ovary, P450-17α has been immunolocalized in differentiated theca cells of medium and large antral follicles but not in granulosa cells (Rodgers et al. 1986). In bovine preovulatory follicles, P450-17α mRNA is found in theca cells (Voss & Fortune 1993). Thus, in bovine dominant follicles, the Δ5 pathway appears to be the preferred route leading to the production of androstenedione in theca cells which is then converted to oestradiol by granulosa cells.

Follicular fluid oestradiol concentrations in the first-wave dominant follicle decrease between days 5 and 8 or 5 and 10 (Badinga et al. 1992, Guilbault et al. 1993). This period corresponds to a time of transition when positive growth rate of this follicle also decreases and the follicle enters a static phase of growth. During this growth transition, P450-arom activity remains elevated in spite of declining follicular fluid concentrations of oestradiol and thus it has been suggested that the loss of oestradiol output could be due to a decreased capacity to synthesize androgenic precursors (Badinga et al. 1992). To test this hypothesis, the activities of P450-arom, 3β-HSD and P450-17α enzymes were determined in homogenized granulosa and theca cells of first-wave dominant follicles and were compared with follicular fluid steroid hormone concentrations.

**Materials and Methods**

*Animals and tissue preparation*

Twenty-one Holstein heifers were used in this study, and were maintained under normal husbandry conditions. Detection of oestrus was conducted twice daily to verify that the animals were displaying normal oestrous cycles before use. All animals were examined daily by transrectal ultrasonography (5 MHz linear probe; LS300A, Tokyo Keiki Co., Tokyo, Japan) according to the technique described by Sirois & Fortune (1988). The images were recorded on videotape to monitor the development of follicles greater than 5 mm in diameter. The largest follicle from the first wave was identified by ultrasonography and was removed from the ovary of these cows immediately following flank ovariecotmy with paravertebral and local anaesthesia from days 5 to 18 of the oestrous cycle. The follicular fluid was aspirated and centrifuged at 2000 g for 5 min to remove cell debris. The supernatant was stored at −20 °C for steroid hormone determinations and the pellet was combined with the granulosa cells which were obtained by scraping the intraovarian portion of the follicle with a blunt spatula. The theca interna was dissected away from the surrounding stroma using fine tissue forceps as described by McNatty et al. (1984).

Theca and granulosa cells were transported to the laboratory on ice and processed immediately. Tissues were homogenized in a glass homogenizer in 1 ml 50 mm Tris–HCl buffer, pH 7.5, containing 250 mm sucrose, 25 mm KCl, 5 mm MgCl₂ and 7 mm mercaptoethanol. The homogenate was centrifuged (17,000 g, 1 h, 4 °C; Beckman L7 Ultracentrifuge, Beckman Instruments, Montréal, Québec, Canada) and a whole membrane preparation pelleted. The cytosol was separated from the membranes which were resuspended in 0-5 ml buffer. Tissue fractions were used immediately for enzyme assays. Tissue protein content was estimated using the method of Lowry et al. (1951).

**Classification of follicles**

Ultrasound recordings 48 and 0 h prior to ovariecotmy were used to determine the diameter of the largest follicle. The growth rate was defined as the mean 24-h change in follicular diameter and was expressed in mm per day. However, this calculated growth rate gives a poor indication of the stage of development of the growing follicle. For example, a follicle with an initial diameter of 7 mm and a growth rate of 2 mm over the last 24 h is at an earlier stage of growth than a follicle with an initial diameter of 10 mm which has the same growth rate. Therefore we have adopted a growth index which considers the stage of development as well as the growth rate to classify dominant follicles. The growth index was calculated according to the following equation:

\[
growth\ index = \frac{[A - B/2]}{B} \times 100
\]

where A and B are the diameter of the follicle measured at 0 and 48 h prior to ovariecotmy respectively. Thus, the growth rate (A − B/2) is divided by the initial diameter B and the ratio is expressed as a percentage. A similar result was obtained when growth rate was calculated from the slope of a line of regression from measurements taken at −48, −24 and 0 h. Follicles were then divided into three classes according to their growth index and evaluation of their state of dominance. During the early luteal phase a follicle was considered dominant when its size was larger than that of other follicles from the same cohort. Later in the cycle, a follicle was considered no longer dominant when another cohort of follicles >5 mm was visualized. Follicles with a growth index value >10% were all dominant and were thus classified as growing-dominant.
Follicles with a growth index value <10% were classified as non-growing and further subdivided into non-growing-dominant or non-growing-non-dominant. The 10% cut-off interval used in the classification of follicles was chosen since it reflects the margin of error inherent in an ultrasound measurement of follicle diameter.

Steroid hormone and enzyme assays

Follicular fluid was extracted and separated by chromatography on C-18 and LH-20 columns as previously described (Bélanger et al. 1990). Quantification of oestradiol, oestrone, pregnenolone, progesterone and testosterone were performed by RIA as previously reported (Bélanger et al. 1981, 1990). A low plasma steroid pool was extracted in parallel with follicular fluid samples to estimate the lower limit of sensitivity of the assay system. The lower limits were the following: oestradiol and oestrone (185 pmol/l), pregnenolone (1-6 nmol/l), progesterone and testosterone (3-5 nmol/l). Intra- and interassay coefficients of variation for the measurement of these steroid hormones were 7–9% and 13–20% respectively.

Radiometric assays of steroidogenic enzymes were performed as previously described (Cooke et al. 1993). Aliquots of the granulosa or theca membranes or cytosol (range 1–20 µg protein/ml of incubation volume) were added to Tris–HCl buffer, pH 7-5 (3 ml), containing the steroid substrate (pregnenolone, 10⁻⁷; progesterone, 10⁻⁸; DHEA, 10⁻⁶; testosterone, 10⁻⁹ M, 40 000 c.p.m. ³H) and appropriate nicotinamide cofactor(s) (0-25 mm NADPH and/or NAD⁺) at 37 °C in a recirculating water bath. At intervals of 30, 60 and 90 min, 1 ml of the incubation medium was withdrawn and transferred to glass screw-cap test tubes containing ethyl acetate (10 ml) and 30 µg of each of the appropriate carrier steroids (pregnenolone, progesterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, DHEA, 4-androstenedione, 5-androstenediol, testosterone, 20α-hydroxyprogesterone) and 10 µg oestrone and oestradiol. The tubes were vortexed to terminate the reactions and extract the steroids and then centrifuged to achieve phase separation (800 × g, 10 min). The organic phase was transferred to conical tubes and evaporated under a stream of N₂ at 35 °C. The residue was applied to plastic-coated thin-layer plates and developed in a two-dimensional system to separate the steroids (Hudson et al. 1984). The first dimension was chloroform-acetone (9:1, v/v) and the second dimension was hexane-ethyl acetate (5:3:5, v/v, run twice). Carrier steroids were visualized by u.v. illumination and exposure to iodine vapour. The regions corresponding to the carrier steroids were excised and quantified by scintillation counting.

Optimal conditions with respect to substrate concentration, protein concentration, linearity over time and substrate utilization were established in preliminary studies in order to allow accurate assessments of specific enzyme activities. The metabolism of pregnenolone required the addition of NADPH and NAD⁺ and was used to estimate P450-17α activity by combining the quantities of 17-hydroxypregnenolone, 17-hydroxyprogesterone, DHEA and androstenedione. The steroids 5-androstenediol, testosterone and oestrogens were produced in negligible quantities in these incubations and were omitted from the calculation. Assessments of 3β-HSD activity were expressed as the amount of 4-ene 3-oxo steroids produced in pregnenolone incubations and verified by the conversion of DHEA to 4-androstenedione in the presence of NAD⁺, using Tris–HCl buffer, pH 8-4. P450-arom activity was measured using testosterone as substrate (1 nm) in the presence of NADPH. Cytosols were used for assessments of 20α-hydroxysteroid dehydrogenase activity using progesterone as substrate but none was detected in cytosols from either granulosa or theca. Reaction rates were determined by linear regression analysis calculated from the three time-points and for all the enzyme activities the correlation coefficient (r) was always greater than 0.96. Enzyme activities were expressed as pmol/min per mg protein.

Statistical analysis

Quadratic fit analyses (SAS Institute Inc. 1989) of growth rate versus follicular fluid oestradiol concentration were performed. Mean follicular fluid steroid concentrations and membrane steroidogenic enzyme activities were compared between groups using the Kruskal–Wallis ANOVA on ranks and Dunn’s test (SAS Institute Inc. 1989). Linear regression analysis was performed between the dependent variable oestradiol and each explanatory variable (Draper & Smith 1981). Since the relationship between the two variables did not appear to be homogeneous between groups, regression equations were estimated separately for each group instead of using a global multiple regression model. The Pearson coefficient of correlation (r) was used to test the hypothesis that the correlation differed from 0 with a P value <0.05.

Results

Using the classification method described above, seven follicles were considered growing-dominant (day 5–6), eight follicles were non-growing-dominant (day 7–10) and six follicles were non-growing-non-dominant (day 10–18). The mean growth index was 28.6 ± 6.4% for growing-dominant follicles, 5.6 ± 1.0% for non-growing-dominant follicles and -5.4 ± 2.1% for non-growing-non-dominant follicles. There was a good correlation between follicular fluid oestradiol concentration and growth index (quadratic fit correlation r²=0.98; P<0.001) (Fig. 1).

The mean concentrations of steroid metabolites measured in follicular fluid are show in Table 1. Differences between groups were seen for oestradiol, oestrone

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and progesterone. Oestradiol concentrations were significantly higher in growing-dominant follicles compared with the non-growing-dominant and non-growing-non-dominant follicles. Oestrone concentrations were significantly higher in growing-dominant follicles compared with the non-growing-non-dominant follicles. Progesterone concentrations were significantly higher in the non-growing-non-dominant follicles compared with the growing-dominant follicles. The mean value of enzyme activities are shown in Table 2. Mean values for P450–17α and 3β-HSD activities were not significantly different between groups. P450-arom activity was significantly lower in non-growing-non-dominant follicles compared with growing-dominant and non-growing-dominant follicles.

In the growing-dominant group, the follicular fluid oestradiol concentration was not significantly correlated with granulosa P450-arom activity and oestradiol was not significantly correlated with granulosa or theca 3β-HSD activity (Fig. 2). There was an apparent positive

![Figure 1](image)

**Figure 1** Quadratic correlation between follicular fluid (FF) oestradiol and growth index of combined growing-dominant, non-growing-dominant and non-growing-non-dominant follicles (n=21). Growth index calculations are given in Materials and Methods. The coefficient of determination (r²) was 0.98 (P<0.001).

<table>
<thead>
<tr>
<th>FF steroid concentration</th>
<th>GD</th>
<th>NGD</th>
<th>NGND</th>
<th>Kruskal–Wallis ANOVA on ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol</td>
<td>511±98a</td>
<td>136 ± 16b</td>
<td>20 ± 11b</td>
<td>P=0.02</td>
</tr>
<tr>
<td>Oestrone</td>
<td>57±18a</td>
<td>16 ± 6a</td>
<td>4 ± 0.8a</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>35 ± 9</td>
<td>7 ± 0.9a</td>
<td>37 ± 13</td>
<td>P=0.06</td>
</tr>
<tr>
<td>DHEA</td>
<td>20 ± 6</td>
<td>3 ± 0.4a</td>
<td>4 ± 0.8a</td>
<td>P=0.07</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>5 ± 1</td>
<td>2 ± 0.3b</td>
<td>3 ± 0.7a</td>
<td>P=0.06</td>
</tr>
<tr>
<td>Progesterone</td>
<td>95±14a</td>
<td>119 ± 16ab</td>
<td>530 ± 200b</td>
<td>P=0.03</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>74±32</td>
<td>28 ± 17</td>
<td>55 ± 9</td>
<td>P=0.06</td>
</tr>
<tr>
<td>Testosterone</td>
<td>36±14</td>
<td>16 ± 6</td>
<td>9 ± 2</td>
<td>P=0.33</td>
</tr>
</tbody>
</table>

Values with different superscript letters are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>GD</th>
<th>NGD</th>
<th>NGND</th>
<th>Kruskal–Wallis ANOVA on ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450–17α (theca)</td>
<td>42 ± 11</td>
<td>54 ± 14</td>
<td>44 ± 18</td>
<td>P=0.90</td>
</tr>
<tr>
<td>3β-HSD (theca)</td>
<td>19 ± 7</td>
<td>77 ± 22</td>
<td>40 ± 19</td>
<td>P=0.14</td>
</tr>
<tr>
<td>3β-HSD (granulosa)</td>
<td>34 ± 11</td>
<td>55 ± 15</td>
<td>66 ± 29</td>
<td>P=0.58</td>
</tr>
<tr>
<td>P450-arom (granulosa)</td>
<td>0.8±0.2a</td>
<td>0.4±0.1a</td>
<td>0.1±0.1b</td>
<td>P=0.04</td>
</tr>
</tbody>
</table>

Values with different superscript letters are significantly different (P<0.05).

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**Table 1** Mean concentrations of steroid metabolites ± S.E.M. in nmol/l, measured in follicular fluid (FF) of growing-dominant (GD, n=7), non-growing-dominant (NGD, n=8), and non-growing-non-dominant (NGND, n=6) follicles. In the GD group, aberrant values for oestradiol (2135 nmol/l) and progesterone (3106 nmol/l) from a single animal were excluded from the calculation of the mean.

**Table 2** Mean values ± S.E.M. of enzyme activities in pmol/min per mg protein, measured in granulosa and theca membranes of growing-dominant (GD, n=6), non-growing-dominant (NGD, n=8), and non-growing-non-dominant (NGND, n=6) follicles.

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correlation between oestradiol and theca P450–17α activity, but this was due to one day-5 animal which had relatively high values for oestradiol (2135 nmol/l) and P450–17α activity (197 pmol/min per mg protein). When this animal was excluded from the analysis the correlation between oestradiol and P450–17α activity was no longer significant (Fig. 2). Similarly, the follicular fluid pregnenolone concentration was not correlated with theca P450–17α activity (r=0.7) or with granulosa or theca 3β-HSD activity (P=0.9). However, pregnenolone and oestradiol were positively correlated in this group (r=0.81, P=0.05).

In the non-growing-dominant group, oestradiol was not significantly correlated with granulosa P450-arom activity or theca P450–17α activity (Fig. 3). However, oestradiol was negatively correlated with theca 3β-HSD activity (P=0.03) and also a negative correlation trend (P=0.1) was found with granulosa 3β-HSD activity (Fig. 3). Similarly to oestradiol, pregnenolone was not correlated with theca P450–17α activity (r=0.16, P=0.7) and was negatively correlated with granulosa 3β-HSD activity (r=−0.72, P=0.04) and probably so with theca 3β-HSD activity (r=−0.64, P=0.08). Pregnenolone was not significantly correlated with progesterone concentrations (P=0.26). Pregnenolone and oestradiol were not significantly correlated although a positive correlation trend seemed to exist (r=0.6, P=0.1).

When comparing means between groups, progesterone concentrations were significantly higher in the non-growing-non-dominant follicle group (Table 1).
However, correlations between the progesterone levels and P450–17α and 3β-HSD activities measured in this group were not significant (P=0.37, 0.18 and 0.16 for P450–17α, granulosa 3β-HSD and theca 3β-HSD respectively). Furthermore, oestradiol was not significantly correlated with P450-arom or 3β-HSD activities measured in granulosa cells (Fig. 4). However, oestradiol was positively correlated with theca P450–17α and 3β-HSD activities (Fig. 4). In addition, pregnenolone was negatively correlated with theca P450–17α and 3β-HSD activities (r= -0.83, P=0.04 and r= -0.81, P=0.04). In this group, pregnenolone and oestradiol were not significantly correlated but a negative correlation trend seemed to exist (r= -0.6, P=0.1). Interestingly, the two animals of this group that presented the highest concentration of oestradiol (74 and 27 nmol/l) had the lowest concentration of pregnenolone (3 nmol/l compared with values of 72, 39, 18 and 84 nmol/l for the remaining animals of the group) and also exhibited relatively high theca P450–17α (121 and 43 pmol/min per mg protein) and theca 3β-HSD (116 and 90 pmol/min per mg protein) activities. Finally, there was a significant positive correlation between oestradiol and testosterone only in the non-growing-non-dominant group (r=0.81, P=0.05).

Discussion

The experiments described herein have investigated the possibility that oestradiol biosynthesis in the bovine luteal
phase dominant follicle is regulated at the level of P450-arom, P450–17α and 3β-HSD enzymes. During dominance, oestradiol production was limited by the supply of pregnenolone rather than changes in the level of activity of these steroidogenic enzymes. When dominance was lost, oestradiol production was limited by pregnenolone supply and by the activity of theca P450–17α and 3β-HSD enzymes.

It might be argued that measurements of concentrations of steroids in the follicular fluid represent accumulation rather than active secretion and may not reflect the synthetic capacity of the follicle cells at the time the follicle was isolated. However, several studies indicate that the follicular fluid compartment is dynamic and that changes in follicular fluid steroid concentrations occur in parallel with changes in peripheral blood. A transient increase in follicular fluid progesterone lasting 6 h was observed immediately following the LH surge (Dieleman et al. 1983) which corresponds to a transient increase in progesterone measured in ovarian venous blood (Walters & Schallenberger 1984). The concentration of oestradiol in both the fluid of the ovulatory follicle and peripheral blood decrease concurrently following the LH surge (Dieleman et al. 1983, 1986). The decline in follicular fluid oestradiol concentrations observed in the present study coincides with the terminal decline of the post-ovulatory oestradiol surge measured in plasma (Dieleman et al. 1986, Badinga et al. 1992). It is therefore reasonable to suggest that follicular fluid steroid measurements are a good indication of active secretion.
In the present study, when comparing means between groups, follicular fluid oestradiol concentrations were significantly lower in non-growing-dominant follicles compared with growing-dominant follicles whereas P450-arom activity was not significantly different between these two groups (Tables 1 and 2). These findings are in agreement with Badinga et al. (1992) who also observed a decrease in mean oestradiol production in dominant follicles between day 5 and day 8 despite sustained aromatase activity. In preovulatory follicles, a similar observation was reported when the follicular fluid concentration of oestradiol was shown to decrease within hours of the LH surge although aromatase activity remained elevated (Dieleman et al. 1983, Dieleman & Blankenstein 1984). In the present study, regression analysis gave a better indication of the relationship between these two variables (Figs 2–4). Follicular fluid levels of oestradiol were not correlated with P450-arom activity at any stage of growth examined, indicating that this enzyme is not the rate-limiting step for oestradiol production in these follicles. Furthermore, in rat ovarian follicles, the biosynthesis of oestradiol was not correlated with either P450-arom mRNA or enzyme protein (Hickey et al. 1988). Taken together, these findings support our hypothesis that the decline in follicular fluid oestradiol levels is not regulated at the aromatase step.

In growing-dominant follicles, oestradiol and pregnenolone were positively correlated but neither was significantly correlated with P450–17α or 3β-HSD activities. Thus, in growing-dominant follicles, any controlling influence on oestradiol output would appear to occur prior to pregnenolone biosynthesis since there is no limitation along the steroidogenic pathway for the conversion of pregnenolone to oestradiol.

Badinga et al. (1992) suggested that a decreased capacity to produce androgen precursor could be responsible for the decline in oestradiol production seen in first-wave dominant follicles between days 5 and 8. This time represents a transition period when the dominant follicle ceases to grow but remains dominant (Savio et al. 1988, Stiros & Fortune 1988). Our studies show that in non-growing-dominant follicles, the enzymes that are required for the conversion of pregnenolone to androgen precursor are either not correlated (P450–17α) or are negatively correlated (3β-HSD) with oestradiol, suggesting that these enzyme activities cannot be rate-limiting for oestradiol biosynthesis in the non-growing-dominant follicle. The findings that pregnenolone was negatively correlated with 3β-HSD activity in granulosa and that oestradiol was also negatively correlated with 3β-HSD activity in theca (Fig. 3) suggest that pregnenolone may be metabolized to a steroid other than oestradiol. The obvious candidate would be progesterone; however, there was no correlation between progesterone and pregnenolone in this group. Thus, it would appear that in non-growing-dominant follicles, pregnenolone is still metabolized primarily to oestradiol although at a lower capacity. Therefore, it is possible that pregnenolone synthesis is switched off in non-growing-dominant follicles and that any residual pregnenolone is still being metabolized to oestradiol. Hence in non-growing–dominant follicles, steroidogenesis is being negatively regulated at a point prior to pregnenolone biosynthesis.

In non-growing–non-dominant follicles, the predominance of progesterone in follicular fluid cannot be attributed to an increase in the activity of 3β-HSD or to a decrease in the activity of P450–17α since correlations between progesterone and these enzyme activities were poor. It is possible that progesterone accumulates because it is a less preferred substrate for P450–17α (Lacroix et al. 1974, Fortune 1986) and, once formed, would be metabolized relatively slowly. Alternatively, progesterone metabolism to other steroids may be inhibited or possibly progesterone is being absorbed from sources outside these follicles. In contrast, oestradiol was positively and significantly correlated with P450–17α and 3β-HSD activities in theca and, correspondingly, pregnenolone levels were negatively correlated with these enzymes. In these follicles, the oestradiol concentration was lower than in the growing–dominant group (Table 1) and was positively correlated with testosterone. Taken together, these findings suggest that in non-growing–non-dominant follicles there are at least two confounding effects that limit oestradiol production. First, pregnenolone supply is limited and residual pregnenolone is converted to oestradiol by theca P450–17α and 3β-HSD and granulosa P450–arom activities. Secondly, theca P450–17α and 3β-HSD activities become rate-limiting for oestradiol and testosterone; although this is not evident from mean enzyme activities (Table 1), significant positive correlations between oestradiol and these enzymes were evident (Fig. 4). The positive correlation between oestradiol and P450–17α observed in non-growing–non-dominant follicles, might be due to a decrease in the amount of this enzyme since Xu et al. (1995a) have shown that mRNA levels of theca P450–17α are lower on days 6–10 compared with day 4. These two confounding effects were detected using regression analysis and would not have been observed if only the group means had been considered.

Therefore, P450-arom, P450–17α and 3β-HSD activities are not rate-limiting for oestradiol biosynthesis in luteal phase dominant follicles. Consequently, oestradiol production must be controlled by some step earlier in the steroidogenic pathway.

The mechanisms controlling oestradiol secretion in a differentiating tissue such as the ovarian follicle are complex and involve molecular interactions at several subcellular levels. The gonadotrophins, FSH and LH, must activate their receptors, steroid precursors must be mobilized and steroidogenic enzymes must come into play. Precursor availability may play a major role in regulating oestradiol output. It is probable that an earlier
step in the steroidogenic pathway, such as cholesterol side-chain cleavage activity (P450 scc), intracellular cholesterol transport or activation of the newly discovered steroidogenic acute regulatory protein (Clark et al. 1994) would present appropriate points for the regulation of steroidogenesis. In this regard, the amount of enzyme protein and the activity of the cholesterol side-chain cleavage system in freshly isolated bovine granulosa cells have also been shown to increase following treatment with cyclic AMP, the presumed mediator of gonadotrophin action (Funkenstein et al. 1983). Furthermore, Xu et al. (1995a,b) have shown that mRNA levels for P450 scc and LH receptor decrease in granulosa and theca of first-wave dominant follicles between days 4 and 8, which corresponds to the time at which we have shown that pregnenolone supply is diminishing.

It has been shown that plasma oestradiol is increased when LH pulse frequency is maintained at an elevated level (Stock & Fortune 1993) and plasma concentrations of oestradiol increase following repeated small injections of gonadotrophin-releasing hormone (Glencross 1987). In the present study, follicles were collected from day 5 onwards, a period when progesterone reaches luteal phase levels and LH pulse frequency begins to decrease (Rahe et al. 1980, Walters et al. 1984). The availability of pregnenolone precursor for further metabolism could thus be sufficient to insufficient LH support as the first-wave dominant follicle reaches maximal size. The regulation of oestrogen production could also depend on signal transduction following the binding of LH to its receptor system, since LH stimulation of theca from large follicles collected during the follicular phase has been shown to release different amounts of steroids in spite of constant LH receptor content (McNatty et al. 1985). Therefore, sustained LH support may be important for the immediate supply of steroid precursors that will be metabolized further by 3β-HSD, P450-17α and P450-arom.

In summary, this study has shown that the regulation of oestradiol output of luteal phase dominant follicles occurs at a different level from 3β-HSD, P450-17α or P450-arom enzyme activities. Cholesterol side-chain cleavage activity and/or intracellular cholesterol transport are postulated to be the rate-limiting steps. Studies are in progress to determine if events that lead to pregnenolone biosynthesis may be regulating oestradiol production.

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