Changes in oestrogen-synthesizing ability of preovulatory bovine follicles relative to the peak of LH

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Summary. Preovulatory bovine follicles ($n = 28$) were collected at different times after the onset of standing oestrus until shortly before ovulation. In-vitro conversion of tritiated androstenedione in the presence of NADPH by homogenates of the follicular wall was compared in phases relative to the LH peak. During phase 0 (before the LH surge) conversion into oestradiol-17 β was high and production of oestrone was about 8-fold lower. During phases 1 (0–6 h after the LH peak) and 2A (6–14 h after the LH peak) the production of oestradiol and oestrone remained constant; the percentage of remaining androstenedione increased. In phase 2B (14–20 h after the LH peak) conversion into oestradiol and oestrone had decreased to about one third correlating with a higher percentage of remaining androstenedione. In phase 3 (20 h after the LH peak until ovulation) conversion into oestradiol and oestrone remained constant. The ratio between the production of oestrone and oestradiol remained constant throughout the phases of preovulatory development (0–13), indicating a concurrent inhibition of aromatase and 17 β -hydroxysteroid dehydrogenase activities. Conversion into 19-hydroxyandrostenedione showed a pattern similar to that of oestradiol, and testosterone was produced in minute quantities. The results indicate that in preovulatory bovine follicles eventual inhibition of aromatization takes place at about 14 h after the preovulatory LH peak.

Introduction

In general the preovulatory peak of luteinizing hormone (LH) is believed to terminate oestradiol synthesis by preovulatory follicles (sheep: Moor, 1974; pig: Ainsworth, Tsang, Downey, Marcus & Armstrong, 1980; cow: Staigmiller, England, Webb, Short & Bellows, 1982). In the cow, the oestradiol concentration in the fluid of preovulatory follicles decreases about 6 h after the preovulatory LH peak (Dieleman, Kruip, Fontijne, de Jong & van der Weyden, 1983b), and the androstenedione concentration in the follicular fluid falls sharply, coinciding with the LH peak (Dieleman, Bevers, Poortman & van Tol, 1983a). This indicates that oestradiol synthesis *in vivo* is terminated by an immediate inhibitory action of the LH surge on androgen production, causing depletion of the aromatase substrate. However, inhibition of this enzyme complex appeared during phase 2 of the development of preovulatory follicles (Dieleman *et al.*, 1983b), as indicated by the continuous increase of the ratio of oestrone and androstenedione concentrations in the follicular fluid until 14 h after the LH peak (Dieleman *et al.*, 1983a).

In the present study, therefore, we investigated whether inhibition of the aromatizing activity does occur in the cow at about 14 h after the preovulatory LH peak.

Materials and Methods

Collection of follicles. The ovaries from normally cyclic Dutch-Friesian heifers ($N = 18$) and cows ($N = 11$) were collected at known times after the onset of oestrus until ovulation. The housing of the

animals, assessment of the luteal function by estimating the progesterone concentration in the peripheral blood with a rapid radioimmunoassay (RIA) twice a day and detection of oestrus were as described previously (Dieleman *et al.*, 1983b), as were recovery of the ovaries by ovariectomy, collection of blood samples and RIA of LH in plasma. Immediately after ovariectomy the ovaries were put on crushed ice. The diameters of the largest follicles present were measured with callipers; usually, the diameter of the second largest follicle was equal to or less than one-half of that of the largest follicle. The largest follicle, therefore, was considered to be the preovulatory follicle (Dufour, Whitmore, Ginther & Casida, 1972); moreover, the second largest follicle was almost always atretic (S. J. Dieleman, unpublished data). The fluid of the preovulatory follicles was gently aspirated and small pieces of the follicular wall were fixed in Bouin's fluid and embedded in paraffin wax; sections of 7 μm were stained with haematoxylin–eosin and periodic acid–Schiff's reagent. On the basis of histological examination the follicles (one per animal) used in this study were designated as non-atretic according to the criteria described by Kruip & Dieleman (1982).

Chemicals. When not specified, chemicals were of analytical reagent grade and obtained from Merck AG, Darmstadt, West Germany; organic solvents were distilled just before use. $[7(n)\text{-}^3\text{H}]$ -Androstenedione (sp. act. 0.303 PBq/mol) which was purified by thin-layer chromatography (t.l.c.), $[4\text{-}^{14}\text{C}]$ androstenedione (sp. act. 1.96 TBq/mol), $[4\text{-}^{14}\text{C}]$ testosterone (sp. act. 2.15 TBq/mol), $[4\text{-}^{14}\text{C}]$ oestrone (sp. act. 2.06 TBq/mol) and $[4\text{-}^{14}\text{C}]$ oestradiol (sp. act. 2.04 TBq/mol) were obtained from Amersham International Ltd, Bucks, U.K., and $[6,7\text{-}^3\text{H}]$ 19-hydroxyandrostenedione (sp. act. 1.67 PBq/mol) from New England Nuclear, Boston, MA, U.S.A. The ^{14}C -tracers were subjected to routine checks for purity by t.l.c. and, if necessary, purified by filtration on Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Steroids were purchased from Makor Chemicals Ltd (Jerusalem, Israel) and Steraloids Inc. (Wilton, NH, U.S.A.). Aqueous samples for radioactivity measurements were dispersed in a xylene-based mixture and counted as described previously (Dieleman *et al.*, 1983b). Samples in organic solvents were dissolved in Instafluor II (Packard Instruments Company, Downers Grove, IL, U.S.A.) and counted in a Tricarb counter (460 CD; Packard Instruments Company) and corrected for quenching by applying the external standard ratio method with four quench-correction curves.

Incubation. After excising the loosely embedded follicular wall from the ovary, the tissue was weighed and subsequently homogenized at 0°C in 13 ml Tris–HCl buffer (0.1 M, pH 7.4) with mannitol (0.25 M). The mean interval from ovariectomy until homogenization was 22.2 ± 6.3 (s.d.) min ($n = 29$). The homogenates were centrifuged at 900 g for 20 min at 4°C and the supernatant was used for incubation. The incubation mixture consisted of 0.5 ml propyleneglycol containing about 0.148 MBq $[7(n)\text{-}^3\text{H}]$ androstenedione and 3 ml supernatant, to which was added 0.5 ml of a solution of NADPH (Boehringer, Mannheim, West Germany) in Tris–HCl–mannitol buffer (final concentration of NADPH 0.2 mM). The incubations were carried out under continuous shaking at 39°C in an air atmosphere. After 30, 60 and 120 min, 1 ml samples were pipetted from the incubation mixture and the enzyme reactions were stopped by the addition of 3 ml dichloromethane; thereafter the fractions were stored at -25°C . Blank incubations with 3 ml Tris–HCl–mannitol buffer instead of homogenate showed no conversion of androstenedione after 30, 60 and 120 min incubation ($n = 4$) respectively.

Extraction and processing. After 4-fold extraction with dichloromethane (recovery $98.28 \pm 0.59\%$, $n = 87$), a mixture of ^{14}C -labelled steroids (androstenedione, testosterone, oestrone and oestradiol, about 1.8, 0.8, 0.9 and 5.0 kBq respectively) was added in a known proportion in 10 μl for recovery and recrystallization purposes. Subsequently, the residues after evaporation under N_2 were subjected to filtration on Sephadex LH-20 (6.0 g in a reusable glass column, i.d. 9 mm) eluted with benzene (Uvasol; Merck AG):dichloromethane:methanol (60:35:5, by vol.), after which the fractions containing androstenedione, oestrone and oestradiol were further purified by recrystallization up to a constant ratio of $^3\text{H}/^{14}\text{C}$ activities. The fraction containing testosterone

and 19-hydroxylated androgens was purified by column chromatography (c.l.c.) on Celite 545 (Serva Feinbiochemica, Heidelberg, West Germany) as described by Van Landeghem *et al.* (1981); the columns were developed with a discontinuous gradient of ethylacetate in *iso*-octane (1–100%, v/v). To the respective Celite fractions, 50 µg reference testosterone, 19-hydroxyandrostenedione and 19-hydroxytestosterone were added. The testosterone fraction was acetylated (1 h at 60°C in a closed reaction vial) by dissolving it in 100 µl dry pyridine to which was added 200 µl acetic anhydride. The three steroid fractions were further purified by t.l.c. (dichloromethane : methanol, 97 : 3, v/v). Conversion percentages of oestrone and oestradiol, and the percentage of androstenedione remaining, were calculated from the ^3H activity of the pure steroids in proportion to the total ^3H activity after extraction and corrected for recovery. Those of testosterone were estimated by correcting the ^3H activity of the testosterone–acetate fraction after t.l.c. for recovery and the relative purity (the proportion of ^3H activity of the steroid after and before recrystallization; $74.1 \pm 18.2\%$, $n = 15$). Conversion into 19-hydroxyandrostenedione was calculated by correcting the ^3H activity after t.l.c. for the mean recovery ($60.5 \pm 12.9\%$, $n = 7$) as determined by performing the purification procedure with the 6,7- ^3H -labelled product, and for the relative purity ($94.1 \pm 2.7\%$, $n = 15$). The coefficient of variation due to statistical errors in measurements of radioactivity was <4 and $<15\%$ for percentage conversions of >1 and <0.5 respectively.

Statistical analysis. The paired *t* test (two-tailed) was used to compare the means of two samples. Differences between the means of samples in grouped data were tested for significance by analysis of variance according to Scheffé (1959).

Results

The time when the maximum LH level was recorded in peripheral blood was defined as the LH peak. For animals kept in circumstances similar to those in this study, the mean $\pm$ s.d. intervals between the onset of oestrus, i.e. first standing oestrus, and the onset of the LH surge (first elevated concentration of LH followed by higher values) and between the maximum LH value and ovulation, have been reported previously to be 1.73 ± 2.35 h and 24 ± 1.4 h respectively (Dieleman *et al.*, 1983b).

Before the LH surge (phase 0) 8 follicles were collected; the mean ($\pm$ s.e.m.) diameter and weight of the follicular wall were 17.9 ± 0.9 mm and 345 ± 40 mg respectively. After the LH peak 20 follicles were collected: 5 at 0–6 h after the LH peak (phase 1; size and weight: 16.8 ± 0.9 mm and 284 ± 40 mg respectively), 6 at 6–14 h (phase 2A; 17.0 ± 0.8 mm, 353 ± 48 mg), 5 at 14–20 h (phase 2B; 18.3 ± 0.6 mm, 380 ± 30 mg) and 4 between 20 h and ovulation (phase 3; 18.5 ± 0.9 mm, 486 ± 22 mg). In one heifer (16 mm, 209 mg) ovulation had taken place shortly before ovariectomy, and the interval after the LH peak was 23 h. There was no difference in size or weight of the wall of follicles originating from heifers or cows, and the diameter varied from 15 to 22 mm independently of the stage of preovulatory development. Analysis of variance proved the apparent increase in weight of the follicular wall to be non-significant.

Conversion of [7(n)- ^3H]androstenedione into oestradiol, oestrone and intermediary steroids

Text-figure 1 shows the mean conversion percentage of oestradiol-17 β and the percentage of remaining androstenedione after incubation of homogenates of preovulatory follicles in the phases between the onset of oestrus and ovulation with the results of the statistical analysis. The apparently lower conversion percentage of oestradiol 0–14 h after the LH peak was not significantly different from that before the LH peak, except after 60 min incubation. After 14 h the conversion percentage of oestradiol had decreased sharply and remained low until ovulation. After 30, 60 and 120 min incubation the pattern of the percentage of remaining androstenedione (Text-

Text-fig. 1. Conversion percentage of oestradiol-17 β and the percentage of remaining androstenedione after incubation for 30, 60 and 120 min with [7(n)- ^{3} H]androstenedione of homogenates of preovulatory bovine follicles in the phases of onset of oestrus and ovulation. Values are mean $\pm$ s.e.m. for the no. of follicles indicated (n). At each incubation time and hormone, values with a different superscript letter are significantly different ($P < 0.05$). * Values significantly different from those after incubation for 30 min ($P < 0.05$).

fig. 1) showed three significantly different periods: before the LH surge; from 0 to 14 h after the LH peak; from 14 h until ovulation. The increase of the production of oestrone (Table 1) during incubation was significant at phases 0 and 2A ($P < 0.01$ for both). The relative pattern of the conversion percentage of oestrone after incubation for 30, 60 and 120 min was similar to that of oestradiol in the phases between onset of oestrus and ovulation. The pattern of the sum of the conversion percentages of oestradiol and oestrone was similar to that of oestradiol; statistical analysis of these sums produced the same significant differences as observed for oestradiol. Analysis of variance of the ratios between the conversion percentages of oestrone and those of oestradiol in the phases between onset of oestrus and ovulation showed no significant differences; it remained constant also during incubation (overall mean: 0.130 ± 0.005 , $n = 84$).

The one follicle collected shortly after ovulation showed a low conversion percentage of oestradiol after incubation for 30, 60 and 120 min (0.56, 1.00 and 1.51% respectively) and conversion into oestrone was near the detection limit, i.e. $<0.1\%$.

The production of intermediary steroids is summarized in Table 2. The relative pattern of the conversion into 19-hydroxyandrostenedione was similar to that of oestradiol in the phases between onset of oestrus and ovulation. Shortly before ovulation (phase 3) the production of testosterone was higher ($P < 0.05$) than before the LH surge (phase 0). The unidentified steroid fraction mainly showed a mobility on Sephadex LH-20 similar to that of 19-aldehydo-androstenedione, and the presence of 19-hydroxytestosterone could not be demonstrated.

Table 1. Conversion percentage of oestrone after incubation with [7(n)-³H]androstenedione of homogenates of preovulatory bovine follicles in the phases between onset of oestrus and ovulation

Phase	Period* (h)	No. of follicles	Incubation time (min)		
			30	60	120
0	Before	8	4.02 ± 0.56^a	5.31 ± 0.61^a	6.82 ± 0.57^a
1	0-6	5	2.74 ± 0.59	3.62 ± 0.78	4.92 ± 1.25
2A	6-14	6	2.13 ± 0.32	3.16 ± 0.48	4.52 ± 0.72
2B	14-20	5	0.92 ± 0.28	1.19 ± 0.40	1.53 ± 0.61
3	20-25	4	0.71 ± 0.13	0.81 ± 0.11	1.01 ± 0.19

Values are mean $\pm$ s.e.m.

* In relation to LH peak level.

Values in columns with different superscript letters are significantly different (ac: $P < 0.001$; ab, ad, bc: $P < 0.05$; dc: $P < 0.005$).

Table 2. Conversion percentages of 19-hydroxyandrostenedione and testosterone, and the percentage of unidentified steroid after incubation for 120 min with [7(n)-³H]androstenedione of homogenates of preovulatory bovine follicles in the phases between onset of oestrus and ovulation

Phase	Period* (h)	No. of follicles	19-Hydroxy- androstenedione	Testosterone	Unidentified
0	Before	8	10.3 ± 3.7	0.25 ± 0.09	15.1 ± 2.0
1	0-6	5	13.9 ± 5.3	0.32 ± 0.10	13.2 ± 2.1
2A	6-14	6	10.4 ± 4.7	0.59 ± 0.09	14.1 ± 2.6
2B	14-20	5	7.5 ± 1.8	0.68 ± 0.15	9.6 ± 0.6
3	20-25	4	4.3 ± 0.9	0.86 ± 0.23	8.1 ± 0.4

Values are mean $\pm$ s.e.m.

* In relation to LH peak level.

Discussion

Changes in aromatizing ability of preovulatory bovine follicles relative to the preovulatory peak of LH were investigated in an in-vitro system using homogenates of the follicular wall, with tritiated androstenedione as precursor since this steroid is the final androgen produced by the theca interna (Lacroix, Eechaute & Leusen, 1974). Although in this way the in-vivo intercellular contact and intracellular organization are lost, this system does provide quantitative data on the aromatizing ability of the follicles. Separate incubation of granulosa cells may produce variable results, since collection of these cells is seldom quantitative as shown by histological examination of the remaining theca interna (see Ireland & Roche, 1982). The mural granulosa cells adjacent to the basement membrane are particularly liable to remain attached to the theca interna, and these cells may have the highest capacity for aromatization (Hillier, 1981). Moreover, using homogenates of the follicular wall avoided possible deprivation of unknown thecal factors affecting granulosa aromatase activity as suggested by the in-vitro experiments of Lacroix *et al.* (1974) with separated and recombined bovine thecal and granulosa cells. Assuming that the concentrations of steroids in the wall of preovulatory bovine follicles are similar to those in the fluid, as in sheep (Murdoch & Dunn, 1982), the concentration of tritiated androstenedione ($0.122 \mu\text{mol/l}$) used in the present incubation experiments was about 4-fold higher than the concentration of endogenous androstenedione of the wall of the preovulatory follicles before the LH surge (phase 0); its concentration in the fluid of preovulatory bovine follicles during this phase is $1.4 \mu\text{mol/l}$ (Dieleman *et al.*, 1983a), which would give an androstenedione concentration of $0.028 \mu\text{mol/l}$ after correction for the concentration of homogenate during incubation. Hillier, Reichert & Van Hall (1981) showed that the androgen concentration in the fluid of human preovulatory follicles is sufficient to permit maximal aromatase activity of the granulosa cells *in vitro*. Although thecal cells are capable of androgen synthesis *in vitro* without exogenous substrate (pig: Evans, Dobias, King & Armstrong, 1981), endogenous androstenedione production during the present experiments probably did not interfere with the production of tritiated oestrogens, because this production remained constant throughout the period 0 to 14 h after the LH peak, whereas a sharp decrease to one seventh of the androstenedione concentration in the fluid has been observed in follicles during phase 1 (0–6 h after the LH peak: Dieleman *et al.*, 1983a). It is therefore assumed that the sum of the oestradiol and oestrone production in the in-vitro system used reflected the in-vivo changes of the aromatizing ability of the preovulatory follicles.

The high conversion into oestrogens and their production ratio before the LH surge are in accordance with the high oestradiol concentration and the ratio between the concentrations of the two steroids in the fluid of follicles at phase 0 respectively (Dieleman *et al.*, 1983a, b). From 0 to 14 h after the LH peak (phases 1 plus 2A) the aromatizing ability remained constant at a lower level (not significant) than in the preceding phase before the LH surge, but conversion into oestrone was lower and the percentage of remaining androstenedione higher than in phase 0. This may be due to the possible difference in aromatizing capacity of the mural granulosa cells and those bordering the antrum, and to the lag between the times at which LH reaches these cells. Passage of LH from the thecal blood capillaries through the avascular membrana granulosa to the antrum takes about 4 h as indicated by the LH peak in the follicular fluid at 4 h after the peak in the peripheral blood (Dieleman *et al.*, 1983a). Also the aromatase activity may be affected by changes in thecal cell function as indicated by the elevated progesterone concentration and the sharp decline of the androstenedione concentration in the follicular fluid during phase 1 (Dieleman *et al.*, 1983a, b). Saidapur & Greenwald (1979) showed that progesterone administered *in vivo* depresses the oestradiol concentration in the peripheral blood of hamsters. In the cow, in phase 2B (14–20 h after the LH peak) the aromatizing ability had decreased to about one third of that in the preceding phases 1 and 2A. Thereafter in phase 3 it decreased further until ovulation. The one follicle collected shortly after ovulation showed a still lower aromatizing ability, and histological examination of this structure revealed an intact membrana granulosa with a luteinized appearance similar to that of preovulatory follicles at phase 3 (Dieleman *et al.*, 1983b). This is in accordance

with the findings of Henderson & Moon (1979) that bovine granulosa cells show a marked loss of aromatizing ability during luteinization in culture.

The similarity between the pattern of the conversion percentage of 19-hydroxyandrostenedione and those of the oestrogens, the low testosterone production and the absence of detectable 19-hydroxytestosterone suggest that androstenedione serves as major substrate for aromatase, since the first of the three hydroxylations during aromatization occurs at C-19 (Thompson & Siiteri, 1974). Addition of testosterone to the medium of cultured bovine follicles at oestrus did not enhance oestradiol secretion by these follicles (Staigmiller *et al.*, 1982). The finding that the ratio of the conversion percentages of oestrone and oestradiol remained constant throughout the phases of preovulatory development may indicate that the activities of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and aromatase are inhibited simultaneously. On the other hand, 17 β -HSD activity may be limited due to a decrease of substrate, although this seems less likely, since conversion into testosterone remained low even when androstenedione was available to a greater extent as substrate for 17 β -HSD.

The observed pattern of aromatizing ability demonstrated that eventual inhibition of the aromatizing enzyme system takes place in preovulatory bovine follicles at about 14 h after the preovulatory LH peak. This confirms the concept based on changes *in vivo* in the ratio between the concentrations of oestrone and androstenedione in the fluid of preovulatory bovine follicles (Dieleman *et al.*, 1983a). Furthermore, these findings are consistent with the reported lower aromatizing ability of bovine granulosa cells collected 10–15 h after the LH peak than of cells before the LH peak (Fortune & Hansel, 1979). Oestradiol production by preovulatory bovine follicles *in vivo* is probably terminated by an immediate inhibitory action of LH on thecal androstenedione production (Dieleman *et al.*, 1983a), but the effect on the activity of the aromatizing enzyme system takes about 10 h to become evident. It may be assumed that the granulosa LH receptors are saturated at 4 h after the LH peak in the peripheral blood (Dieleman *et al.*, 1983a); Ireland & Roche (1982) reported a 5-fold lower binding capacity for hCG of bovine granulosa cells collected after than before the LH surge. Whether the preovulatory LH peak inhibits aromatization by a direct or indirect action on the aromatizing enzyme systems needs further investigation. Suzuki & Tamaoki (1983) reported an acute decrease of cytochrome P-450 in rat preovulatory ovaries after treatment with hCG, but the activity of NADPH-cytochrome c reductase was not significantly impaired.

Four phases of preovulatory development can be distinguished with regard to changes in the steroidal microenvironment of the bovine oocyte (Dieleman *et al.*, 1983b) which is considered to be mature at phase 3 (Kruip, Cran, Van Beneden & Dieleman, 1983). The results of this study show that one phase (phase 2) can be subdivided according to the eventual inhibition of aromatase.

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