Gonadotrophin responsiveness, aromatase activity and insulin-like growth factor binding protein content of bovine ovarian follicles during the first follicular wave

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The aim of this study was to examine the function of granulosa cells and hormone concentrations in follicular fluid in bovine ovarian follicles during selection of the first dominant follicle. Ovaries were obtained from beef heifers on days 1–5 after ovulation: follicles > 4 mm in diameter were dissected and follicular fluid and granulosa cells were collected from individual follicles. Oestradiol production by granulosa cells after culture with testosterone was used to determine aromatase activity and responsiveness to gonadotrophins was determined by cAMP production after culture with FSH or LH. Concentrations of oestradiol, progesterone and insulin-like growth factor binding proteins (IGFBPs)-4 and -5 were measured in follicular fluid. Follicles were classified as largest or smaller (days 1 and 2), or dominant or subordinate (days 3–5). Aromatase activity was greater in granulosa cells from the largest follicle than in granulosa cells from smaller follicles on

days 1, 3, 4 and 5 ($P < 0.05$). Responsiveness to LH was not detected in granulosa cells on day 1, but from day 2 to day 5 cells from the largest follicle were significantly more responsive than cells from smaller follicles ($P < 0.05$). Responsiveness to FSH was detected in granulosa cells from all follicles from day 1 onwards and did not differ between cells from the largest follicle or smaller follicles on any day. Follicular fluid concentrations of oestradiol and the ratio of oestradiol:progesterone were greater and concentrations of IGFBP-4 and -5 were lower in the largest follicle than in smaller follicles from day 2 to day 5 ($P < 0.05$). In conclusion, selection of the dominant follicle is associated with increased granulosa cell aromatase activity followed by increased cAMP response to LH and follicular fluid oestradiol concentrations, and decreased follicular fluid concentrations of IGFBP-4 and -5 within 2 days after ovulation.

Introduction

The wave-like growth and development of ovarian follicles in cattle was first described by Cupps *et al.* (1959). Subsequent more detailed histological studies revealed that a group of healthy follicles > 4 mm in diameter develops within the ovaries 2 or 3 days after oestrus, but that only one follicle continues to grow until day 10, while the other follicles regress. This process is repeated commencing on day 11 after oestrus, culminating in maturation of the ovulatory follicle (Rajakoski, 1960). Confirmation of these observations came with the development of transrectal ultrasonography, allowing individual follicles to be monitored non-invasively (Pierson and Ginther, 1988; Savio *et al.*, 1988; Sirois and Fortune, 1988). Detailed studies involving examinations at 8 h intervals have demonstrated that a wave of follicles ≥ 3 mm in diameter can be detected emerging over 72 h and that the future

dominant follicle is first evident 6 or 10 h earlier than the largest or second largest subordinate follicle, respectively (Ginther *et al.*, 1997).

Emergence of a follicular wave is preceded by increased peripheral concentrations of FSH (Adams *et al.*, 1992; Sunderland *et al.*, 1994). The peak of FSH is observed on average 1 or 2 days before follicles 4 and 5 mm in diameter are detected by ultrasonography. Subsequently, selection of the dominant follicle appears to be associated with decreasing concentrations of FSH, as treatment of heifers with recombinant FSH delays emergence of the dominant follicle (Adams *et al.*, 1993; Mihm *et al.*, 1997). The selected dominant follicle also develops an increased ability to produce oestradiol. This may be expressed in terms of concentrations of oestradiol in follicular fluid or in plasma from the caudal vena cava, or as the capacity of granulosa cells to produce oestradiol in culture (Bodensteiner *et al.*, 1996; Evans and Fortune, 1997; Ginther *et al.*, 2000). Higher concentrations of oestradiol in follicular fluid of dominant follicles compared with subordinate follicles are evident by day 3 of the oestrous cycle or 1.5 days after emergence of the first follicular wave (Mihm *et al.*, 2000). Aromatase activity

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of granulosa cells (the ability to metabolize androgens to oestradiol) from dominant follicles is significantly greater than that of cells from subordinate follicles on day 5 after oestrus (Badinga *et al.*, 1992), but has not been examined in follicles before day 5.

Although a decrease in circulating concentrations of FSH is required for selection of the dominant follicle, low concentrations of this gonadotrophin are necessary for continuing development of the largest follicle (Gong *et al.*, 1996; Ginther *et al.*, 1999). The dominant follicle also requires LH for growth beyond 7–9 mm in diameter (Gong *et al.*, 1996). The differential expression of receptors for LH and FSH, or their mRNA, in granulosa cells during recruitment and selection has been examined in a number of studies. Xu *et al.* (1995) reported that expression of mRNA for FSH receptors in granulosa cells of healthy follicles was present from day 2 after wave emergence, but did not vary with follicle size. However, mRNA for LH receptors in granulosa cells was expressed in healthy follicles > 9 mm in diameter only and was first observed in dominant follicles collected 4 days after initiation of the follicular wave. Other studies have reported expression of mRNA for LH receptors in healthy follicles between 36 h and 48 h after emergence (Bao *et al.*, 1997), an absence of expression of mRNA for LH receptors in granulosa cells on day 3 after wave emergence (Evans and Fortune, 1997) and differences between the largest and smaller follicles in expression of LH receptors on granulosa cells on day 4, but not on day 2, after ovulation (Bodensteiner *et al.*, 1996). Although these studies differ in their definitions of day of emergence, these results all indicate that increased production of oestradiol by the largest follicle precedes expression of receptors for LH by granulosa cells.

A functional measure of responsiveness of granulosa cells to gonadotrophin stimulation is the ability of these cells to produce cAMP in culture. Using materials from an abattoir, Jolly *et al.* (1994) found that on a per cell basis, bovine granulosa cell cAMP production in response to both LH and FSH stimulation was detectable in follicles ≥ 4 mm in diameter. Responsiveness to LH was low or undetectable in cells from follicles 4–8 mm in diameter; it then increased linearly with increasing follicle diameter ≥ 8 mm. The precise stage of follicular development at which granulosa cells develop functional LH receptors is not known; however, acquisition of these receptors may play an important role in the mechanisms underlying selection of the dominant follicle.

Other intrafollicular factors, including inhibins and insulin-like growth factors (IGFs) and their binding proteins (IGFBPs), have been associated with the processes of dominance and atresia (Hopko-Ireland *et al.*, 1994; de la Sota *et al.*, 1996; Stewart *et al.*, 1996; Mihm *et al.*, 1997; Armstrong *et al.*, 1998). High concentrations of oestradiol in dominant follicles are associated with low concentrations of low molecular mass IGFBPs (Echternkamp *et al.*, 1994; de la Sota *et al.*, 1996; Stewart *et al.*, 1996; Mihm *et al.*, 1997, 2000). These proteins may modulate the actions of FSH

indirectly through their effects on the bioavailability of IGF-I, but it is not clear whether changes occur in the potential dominant follicle before or at the same time as selection.

The aim of the present study was to compare the capacity of granulosa cells to produce oestradiol and to respond to LH or FSH by synthesizing the second messenger cAMP between potential dominant and subordinate follicles during the first 5 days of the oestrous cycle in cows, the period during which the dominant follicle of the first follicular wave is selected. In addition, follicle size and stage of development when granulosa cell cAMP response to LH was first evident was determined. Differences in follicular fluid concentrations of oestradiol, IGFBP-4 and -5 and the ratio of oestradiol:progesterone between the largest and smaller follicles during the first 5 days after ovulation were also characterized.

Materials and Methods

Animals and procedures

All procedures were carried out following approval by the Ruakura Animal Ethics Committee, in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand.

Reproductively mature crossbred beef heifers weighing 368.0 ± 4.6 kg (mean $\pm$ SEM) and aged approximately 1.5 years were grazed on mainly ryegrass and white clover pasture with access to water *ad libitum*. Oestrous cycles of heifers were synchronized using a controlled intravaginal drug-releasing device (CIDRTM; DEC-InterAg, Hamilton) containing 1.9 g progesterone for 10 days and an i.m. injection of 500 μ g of the PGF_{2 α} analogue cloprostenol sodium (Estrumate; Mallinckrodt Veterinary Ltd, Upper Hutt) 6 days after insertion of the device.

The development of ovarian follicles was monitored once a day at the same time of day using transrectal ultrasonography with a 7.5 MHz transducer (Aloka DX210; Medtel, Auckland) commencing on the day after removal of the CIDRTM device. Each day a map was drawn of the size and location of all follicles ≥ 4 mm in diameter on each ovary. The dominant follicle was defined as the largest follicle in the ovary that continued to grow when the other follicles regressed and was ≥ 2 mm greater in diameter than any other follicle. The day of ovulation was defined as the day on which the dominant follicle was no longer observed and was replaced by a new corpus luteum. Day of emergence was defined as the first day after ovulation on which a cohort of follicles ≥ 4 mm in diameter was observed. Day of deviation was defined as the day that the largest follicle was ≥ 2 mm greater in diameter than the second largest follicle. Animals were observed twice a day to detect the time of onset of oestrus, determined by observations of oestrous behaviour with the aid of tailpaint removal (Macmillan *et al.*, 1988).

After the synchronized oestrus, the ovary bearing the dominant follicle was removed by unilateral ovariectomy

on days 4, 5, 6 or 7 after ovulation ($n = 9, 5, 4$ and 3 , respectively). Five days after first ovariectomy, the oestrous cycles of the heifers were re-synchronized using the same protocol and the remaining ovary was collected after death on days 1, 2, 3 or 4 after ovulation ($n = 4, 6, 7$ and 3 , respectively). Heifers were allocated randomly to day of ovariectomy before each round of oestrous synchronization. Previous studies have demonstrated that folliculogenesis during the first 11 days of the oestrous cycle is similar in cows with one or two ovaries (Badinga *et al.*, 1992).

Animals were not fasted before surgery and unilateral ovariectomy was performed with heifers standing in a crush, using caudal epidural anaesthesia with 2% (w/v) lignocaine. Ovaries were removed with an ecraseur as described by Drost *et al.* (1992).

Follicle dissection and cell cultures

After ovariectomy or death, ovaries were placed in Medium A at room temperature. Medium A was Dulbecco's modified Eagle's medium (DMEM: with glucose and L-glutamine, without bicarbonate; Gibco, Grand Island, NY) containing 20 mmol Hepes buffer l^{-1} (Sigma, St Louis, MO), 0.1% (w/v) BSA (Sigma) and 50 μg gentamicin ml^{-1} (Sigma). Within 1 h of collection, all follicles ≥ 5 mm in diameter were identified as closely as possible with those identified by ultrasonography, dissected free from ovarian stroma and their diameters were measured. Follicular diameters reported refer to those measured after dissection. All procedures were carried out at room temperature and as quickly as possible. Follicular fluid was aspirated from each follicle and then frozen rapidly to -20°C for later steroid hormone and IGFBP analysis.

Granulosa cells were collected separately for each follicle, counted and allocated for cell culture as described previously (McNatty *et al.*, 1984; Jolly *et al.*, 1994) for determination of granulosa cell aromatase activity and cAMP response to FSH and LH. Cells were washed and cultured in Medium B, which was Medium A without antibiotic, to which 0.2 mmol 3-isobutyl-1-methylxanthine l^{-1} (IBMX, a phosphodiesterase inhibitor; Sigma) was added. For cAMP studies, washed cells (10^5 cells) in 0.5 ml Medium B were incubated in duplicate or triplicate with either 0.5 ml Medium B alone or with 0.5 ml Medium B + 10 ng oFSH (purified Ovagen; Immuno-Chemical Products Ltd, Auckland; a gift from L. Moore, Wallaceville, New Zealand; biopotency equivalent to 86 000 ng NIDDK-oFSH-17 ml^{-1}) or 100 ng oLH (NIDDK-oLH-I-2) for 45 min at 37°C . The tubes were then heated to 80°C for 15 min and frozen to -20°C until assayed for cAMP by radioimmunoassay. Optimal concentrations of gonadotrophins for maximal cAMP production had been determined previously (F. M. Rhodes, A. J. Peterson and G. Parton, unpublished). Granulosa cell cAMP response was calculated as the difference in cAMP concentrations between samples incubated for 45 min, with and without LH or FSH, and expressed as fmol cAMP produced per 10^5 cells per 45 min.

Zero responsiveness to LH or FSH was defined as ≤ 26.0 fmol cAMP produced per 10^5 cells per 45 min, which was the upper 95% confidence interval for cAMP production in the absence of gonadotrophin.

For determination of aromatase activity, washed cells (10^5 cells) in 0.5 ml Medium B were incubated in duplicate or triplicate with either 0.5 ml Medium B alone or with 1 μg testosterone for 3 h at 37°C as described by McNatty *et al.* (1984) and then snap-frozen and stored at -20°C until assayed for oestradiol. Aromatase activity was determined as the difference in oestradiol concentrations between samples incubated with and without testosterone for 3 h and expressed as ng oestradiol produced per 10^5 cells per 3 h.

Assays for steroid hormones, cAMP and IGFbps

Concentrations of cAMP in the culture medium were determined by radioimmunoassay (McNatty *et al.*, 1990), except that separation of bound from free fractions was by second antibody reaction followed by the addition of 2.5 volumes 5% (w/v) polyethylene glycol 8000 (Union Carbide Corp., Danbury, CT) just before final centrifugation. Cell culture samples were thawed and assayed directly, giving a measure of total (intra- and extracellular) cAMP content. The sensitivity of the assay was 1.88 fmol ml^{-1} . The intra- and interassay coefficients of variation were 11.2% and 12.3%, respectively, for a pooled sample containing 52.8 fmol cAMP ml^{-1} (15 assays).

Concentrations of progesterone were measured in follicular fluid samples using a solid phase, [^{125}I]-label radioimmunoassay (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA). The sensitivity of the assay was 0.04 ng ml^{-1} . The intra-assay coefficients of variation were 6.4% and 6.3% for pooled samples containing 0.45 and 3.02 ng progesterone ml^{-1} , respectively (one assay).

Concentrations of oestradiol in culture medium and follicular fluid were determined using a modified radioimmunoassay as described by Prendiville *et al.* (1995). The sensitivity of the assay was 0.52 pg ml^{-1} . The intra- and interassay coefficients of variation were 14.8% and 15.6%, respectively, for a pooled sample containing 29.2 pg oestradiol ml^{-1} , and 18.1% and 18.7%, respectively, for a pooled sample containing 46.4 pg oestradiol ml^{-1} (five assays).

Western ligand blotting following the method of Hossenlopp *et al.* (1986) was used to visualize the IGFbps in follicular fluid. Samples (2 μl) were subjected to SDS-PAGE under non-reducing conditions and the IGFbps were detected after incubation with iodinated IGF-2 using a previously published protocol (Peterson *et al.*, 1998a). Western immunoblotting using antisera against human IGFBP-4 and -5 (UBI, Lake Placid, NY) and the method reported by Peterson *et al.* (1998a) revealed that the bands of 29–30 kDa and 23–24 kDa crossreacted with both antisera (data not shown). Therefore, the identity of the IGFbps at these molecular masses is uncertain and the results are

presented as the sum of the densities of the two and reported as IGFBP-4 and -5. Analysis of the autoradiographs was performed by densitometry (Molecular Dynamics, Sunnyvale, CA) after standardization of the same bands from a control of bovine follicular fluid run in parallel with each gel (Peterson *et al.*, 1998b). The coefficient of variation for this control was 3.0% across 12 gels.

Statistical analyses

On days 6 and 7 after ovulation, only three and two dominant follicles, respectively, were obtained after ovariectomy; therefore, results are presented for days 1–5 after ovulation only. Data from follicles that were present before ovulation (not in the cohort of newly emerged follicles) were excluded from all analyses. One-way ANOVA was used to determine the significance of differences between the largest follicle and smaller follicles on days 1 and 2 after ovulation, and between dominant and subordinate follicles on days 3, 4 and 5 after ovulation. The largest follicle was the follicle from an ovary with the largest diameter measured after dissection. On day 2, three 'largest' follicles from one heifer were of equal diameter and on day 3, two 'largest' follicles from one heifer were of equal diameter. These were excluded from analyses. Insufficient cell numbers were obtained from some follicles to conduct all the cell cultures, so the numbers of follicles used in analyses varied, as indicated in the tables of results. All data were logarithmically transformed before analysis, but results are presented as arithmetic means $\pm$ SEM.

Results

The mean interval from onset of oestrus to ovulation was 1.1 ± 0.1 days (range 0–2 days, $n = 34$), from oestrus to follicular wave emergence was 1.5 ± 0.1 days (range 1–2 days) and from ovulation to emergence was 0.4 ± 0.1 days (range 0–1 days). These intervals did not differ between the first and second rounds of oestrous synchronization. In the heifers ovariectomized after establishment of the dominant follicle, the interval from oestrus to deviation of the dominant and subordinate follicles was 3.2 ± 0.1 days (range 2–5 days, $n = 27$) and from ovulation to deviation was 2.1 ± 0.14 days (range 1–4 days).

On day 1 after ovulation, diameter of dissected follicles and aromatase activity of granulosa cells were significantly greater in the largest follicle than in smaller follicles ($P < 0.05$; Table 1). Concentrations of oestradiol, IGFBP-4 and -5, the ratio of oestradiol:progesterone and responsiveness of granulosa cells to LH or FSH were all similar in the largest and smaller follicles (Table 1). Responsiveness to LH by granulosa cells from all follicles was below the zero response, whereas granulosa cells from all dissected follicles showed responsiveness to FSH greater than zero responsiveness.

On day 2 after ovulation, the diameter of dissected follicles, follicular fluid concentrations of oestradiol, ratio of oestradiol:progesterone and responsiveness of granulosa

cells to LH were all significantly greater in the largest follicle compared with smaller follicles ($P < 0.05$, Table 1). Follicular fluid concentrations of IGFBP-4 and -5 were significantly lower in the largest follicle ($P < 0.05$), but there were no differences in aromatase activity of granulosa cells or in responsiveness to FSH (Table 1). Responsiveness to LH greater than the zero response was observed in granulosa cells from five follicles ≥ 9 mm in diameter (Fig. 1a).

On day 3 after ovulation, diameter of dissected follicles, aromatase activity and responsiveness of granulosa cells to LH, follicular fluid concentrations of oestradiol and ratio of oestradiol:progesterone were all significantly greater and follicular fluid concentrations of IGFBP-4 and -5 were lower in dominant than subordinate follicles ($P < 0.05$, Table 2). There were no significant differences in responsiveness of granulosa cells to FSH (Table 2). Responsiveness to LH was greater than zero in granulosa cells from two follicles 7 mm in diameter and six follicles ≥ 10 mm in diameter and in cells from subordinate as well as dominant follicles (Fig. 1b).

Dominant follicles dissected from ovaries on days 4 and 5 after ovulation differed significantly ($P < 0.05$) from subordinate follicles in all the variables measured, except for responsiveness of granulosa cells to FSH (Table 2).

Discussion

In this study the function of granulosa cells from bovine ovarian follicles from day 1 to day 5 after ovulation, a period that covers selection of the dominant follicle of the first follicle wave, was examined. The most significant finding was that there are significant differences in aromatase activity of granulosa cells between follicles in the bovine ovary by day 1 after ovulation, at about the time when a new cohort of emergent follicles can be detected using ultrasonography. Although the dominant follicle cannot be identified on the basis of size during the early stages of the follicular wave, previous studies have used size as a means of categorizing follicles on day 2 after emergence (Evans and Fortune, 1997), day 2 after ovulation (Bodensteiner *et al.*, 1996) or during the peri-oestrous period (Sunderland *et al.*, 1994). The results of the present study indicate that a difference in size is associated with differences in responsiveness to LH and in capacity to synthesize oestradiol. The latter finding is in agreement with other studies demonstrating increased follicular fluid concentrations of oestradiol in the largest follicle compared with the second largest follicle on day 2 after emergence (Evans and Fortune, 1997) or day 2 after ovulation (Bodensteiner *et al.*, 1996). Mihm *et al.* (2000) reported that by day 1.5 after emergence follicular fluid concentrations of oestradiol are higher in the future dominant follicle than in future subordinate follicles. The early expression of aromatase activity by granulosa cells is in agreement with the findings of Bao *et al.* (1997) in which mRNA expression for cytochrome P450 side-chain cleavage and cytochrome P450 aromatase was present in granulosa cells by 12 h after wave emergence, but also indicates that, on a per cell basis, granulosa cells from the largest follicle present on day 1 after ovulation have greater

Table 1. Characteristics of the largest and smaller ovarian follicles in beef heifers on days 1 and 2 after ovulation

Characteristic	Day 1			Day 2		
	Largest follicles	Smaller follicles	P value ^a	Largest follicles	Smaller follicles	P value ^a
Number of follicles	3–4	6–12		5	7	
Diameter (mm)	10.0 ± 0.4	6.2 ± 0.3	< 0.001	10.8 ± 0.4	7.7 ± 1.5	0.002
Aromatase activity ^b	887 ± 49	499 ± 132	0.040	1390 ± 339	747 ± 201	0.193
IGFBP-4 and -5 ^c	476 ± 112	1199 ± 275	0.146	545 ± 102	1205 ± 200	0.015
Oestradiol in follicular fluid (ng ml ⁻¹)	14.7 ± 1.8	10.7 ± 3.1	0.328	105.6 ± 30.6	15.6 ± 15.2	0.015
Responsiveness to FSH ^d	732 ± 252	664 ± 225	0.625	786 ± 300	934 ± 188	0.459
Responsiveness to LH ^d	6.29 ± 2.34	7.22 ± 1.57	0.996	103.0 ± 28.5	11.0 ± 4.3	0.002
Ratio of oestradiol:progesterone in follicular fluid	0.17 ± 0.04	0.10 ± 0.03	0.182	1.45 ± 0.41	0.17 ± 0.07	0.009

Values are arithmetic means ± SEM.

^aSignificance of difference between largest follicle and smaller follicles, within day.

^bng oestradiol produced per 10⁵ cells in 3 h.

^cInsulin-like growth factor binding proteins (IGFBPs)-4 and -5 expressed in absorbance units.

^dfmol cAMP produced per 10⁵ cells in 45 min.

Fig. 1. Responsiveness to LH of granulosa cells from individual follicles of different diameter collected from beef heifers on (a) day 2 (●: largest follicle; ○: smaller follicle) and (b) day 3 (●: dominant follicle; ○: subordinate follicle) after ovulation. Responsiveness was expressed as fmol cAMP produced per 10⁵ cells in 45 min. The horizontal line demonstrates zero responsiveness to LH (26.0 fmol cAMP per 10⁵ cells).

oestradiol-synthesizing capacity (aromatase activity) than those of smaller follicles. This difference in aromatase activity preceded differences in follicular fluid concentrations of

oestradiol and in the ratio of oestradiol:progesterone in follicular fluid, which were significantly greater in the largest follicle than in smaller follicles on day 2 after ovulation. Differences in aromatase activity of granulosa cells between the largest follicle and smaller follicles were not significantly different on day 2 after ovulation, reflecting a greater variability among follicles at this time. However, cells from the largest follicles had a numerically greater aromatase activity, as demonstrated by concentrations of oestradiol in follicular fluid.

The second important finding of the present study was that significant responsiveness to LH was first detected on day 2 after ovulation, equivalent to day 2.4 after wave emergence and at about the time of deviation of the dominant and subordinate follicles. Synthesis of cAMP in response to LH was significantly greater in granulosa cells from the largest follicle compared with cells from smaller follicles at this time, but was only present at concentrations greater than zero responsiveness in cells from follicles > 9 mm in diameter. These findings contrast with those from studies in which the expression of mRNA for LH receptors in the granulosa cells of follicles was examined. Xu *et al.* (1995) found expression of mRNA for LH receptor from day 4 after emergence only; similarly, Evans and Fortune (1997) did not detect expression of mRNA for LH receptors in granulosa cells of dominant or subordinate follicles on days 2 or 3 after emergence. Measurement of LH receptors using a radioreceptor assay demonstrated the presence of LH receptors on granulosa cells on day 2 after ovulation, but no difference in number of receptors per cell between the largest and second largest follicles (Bodensteiner *et al.*, 1996). The early expression of responsiveness to LH found in the present study compared with studies examining expression of mRNA for LH receptors may reflect differences in the sensitivities of the methods used to detect and quantify the presence of LH

Table 2. Characteristics of the dominant and subordinate ovarian follicles in beef heifers on days 3, 4 and 5 after ovulation

Characteristic	Day 3			Day 4			Day 5		
	Dominant	Subordinates	Pvalue ^a	Dominant	Subordinates	Pvalue ^a	Dominant	Subordinates	Pvalue ^a
Number of follicles	6	7–12		10	8–16		5	2–5	
Diameter (mm)	11.5 ± 0.8	7.7 ± 0.4	<0.001	12.5 ± 0.9	7.2 ± 0.5	<0.001	16.2 ± 0.6	8.4 ± 0.7	<0.001
Aromatase activity ^b	1675 ± 383	435 ± 141	0.009	1635 ± 361	367 ± 240	0.001	1686 ± 388	140 ± 129	0.014
IGFBP-4 and -5 ^c	505 ± 95	1846 ± 358	0.015	1298 ± 370	6877 ± 1257	<0.001	1830 ± 511	11934 ± 2816	0.001
Oestradiol in follicular fluid (ng ml ⁻¹)	97.4 ± 20.3	11.3 ± 5.6	0.009	39.1 ± 8.7	3.4 ± 3.1	<0.001	48.1 ± 12.8	1.2 ± 1.1	0.009
Responsiveness to FSH ^d	606 ± 68	506 ± 114	0.239	931 ± 190	633 ± 188	0.212	1296 ± 355	484 ± 201	0.160
Responsiveness to LH ^d	151.6 ± 64.3	27.9 ± 12.2	0.049	296.1 ± 101.6	71.3 ± 58.4	0.022	1090 ± 336	103 ± 101	0.035
Ratio of oestradiol:progesterone in follicular fluid	1.47 ± 0.32	0.11 ± 0.06	0.019	0.82 ± 0.21	0.05 ± 0.05	<0.001	1.20 ± 0.36	0.01 ± 0.01	0.001

Values are arithmetic means ± SEM.

^aSignificance of difference between dominant and subordinate follicles, within day.

^bng oestradiol produced per 10⁵ cells in 3 h.

^cInsulin-like growth factor binding proteins (IGFBP)₃₋₄ and -5 expressed in absorbance units.

^dfmol cAMP produced per 10⁵ cells in 45 min.

receptors. Measurement of increases in the concentrations of the second messenger cAMP in response to LH are indicative not only of the presence of LH receptors, but also their functional coupling to an intracellular signal transduction mechanism. Differences between studies may also be due to the differences in definitions of the time of emergence of the follicular wave and to variation between animals in the timing of emergence relative to onset of oestrus or ovulation. In the present study, data from a total of 20 animals were used and the interval from oestrus to emergence varied between 1 day and 2 days. In the study of Evans and Fortune (1997), the interval to emergence varied between 0 and 2 days in six animals, whereas the range was 1–3 days in the study of Xu *et al.* (1995). Thus, there may be considerable overlap in the timing of these events among the various studies. In agreement with the current study, Beg *et al.* (2001) reported significantly higher expression of granulosa cell LH receptor mRNA in the largest follicle compared with second largest follicles just before diameter deviation.

On day 3 after ovulation, responsiveness to LH was detected in granulosa cells from both dominant and subordinate follicles > 6 mm in diameter; however, cells from dominant follicles were significantly more responsive than cells from subordinate follicles, in agreement with previous studies examining granulosa cells for LH receptors (Ireland and Roche, 1983; Bodensteiner *et al.*, 1996). Deviation of the dominant and subordinate follicles begins at about day 3 after follicle wave emergence (Ginther *et al.*, 1997), but selection of the dominant follicle is not absolute until day 5 after ovulation, as removal of the largest follicle before this results in the second largest follicle assuming dominance (Ko *et al.*, 1991). The results of the present study support these previous results by demonstrating that more than one follicle has the capacity to respond to LH, although the largest follicle has a significant advantage in terms of cAMP production.

Responsiveness to FSH was present in the granulosa cells of all the dissected follicles > 4 mm in diameter from day 1 after ovulation; however, there was no difference in responsiveness to FSH between granulosa cells from the largest follicle and smaller follicles (days 1 and 2) or between granulosa cells from dominant and subordinate follicles (days 3–5). Similarly, Bodensteiner *et al.* (1996) found no differences in numbers of FSH receptors per granulosa cell between dominant or subordinate follicles on days 2 or 4 after ovulation, but significantly more receptors per follicle on day 4. Ireland and Roche (1983) reported that the capacity of granulosa cells to bind FSH was no different between oestrogen-active or -inactive follicles on day 3 after oestrus, but was significantly higher in cells from oestrogen-active follicles on day 5. The results of the present study indicate that the ability of granulosa cells to bind FSH and produce cAMP is not limiting during the early stages of follicle wave emergence or associated with the mechanism of follicle selection or dominance. The actions of FSH may be attenuated by other factors that allow the dominant

follicle to grow, while suppressing the development of subordinate follicles in an environment of decreasing concentrations of FSH.

Concentrations of IGFBP-4 and -5 were lower in the largest follicle than in smaller follicles from day 2 to day 5 after ovulation. Concentrations in dominant follicles increased at least threefold between day 3 and day 5, whereas concentrations in subordinate follicles increased nearly eightfold over the same period, in agreement with previous studies demonstrating an increase in low molecular mass IGFBPs (IGFBP-2, -4 and -5) in the follicular fluid of bovine follicles with increasing degrees of atresia and decreasing follicular fluid concentrations of oestradiol (Echternkamp *et al.*, 1994; de la Sota *et al.*, 1996; Stewart *et al.*, 1996; Mihm *et al.*, 1997, 2000). Austin *et al.* (2001) reported lower concentrations of IGFBP-2, -4 and -5 in the follicular fluid of the two largest follicles compared with smaller follicles between day 2.6 and day 4.5 after oestrus. These binding proteins appear to be important in the physiological regulation of FSH actions, probably by regulating the bioavailability of IGF-I or IGF-II and FSH-induced oestradiol production by granulosa cells (Gutierrez *et al.*, 1997). It has been demonstrated that lower concentrations of IGFBP-4 in preovulatory and dominant bovine follicles are associated with the presence of an IGFBP-4 protease (Mazerbourg *et al.*, 2000; Rivera and Fortune, 2001); however, it is not known whether this protease is present before selection takes place. The results of the present study confirm an early difference in concentrations of low molecular mass IGFBPs between the largest follicle and smaller follicles, although this difference did not precede differences in oestradiol-synthesizing capacity among follicles.

The increase in the concentrations of IGFBP-4 and -5 in dominant follicles from day 3 to day 5 after ovulation resulted in concentrations on day 5 comparable to those observed in the smallest follicles on day 2. This finding indicates that the bioavailability of IGF-I or IGF-II within the dominant follicle was decreasing during this time. In contrast, during growth of the preovulatory follicle there is a progressive decrease in concentrations of low molecular mass IGFBPs (Echternkamp *et al.*, 1994). The preovulatory follicle never loses its dominance, in contrast to those in previous follicular waves, indicating that loss of dominance is associated with increasing amounts of the low molecular mass IGFBPs. The first dominant follicle is capable of ovulating after induced luteolysis, but loses this capacity between day 6 and day 8 after ovulation, when the second wave of follicles commences emergence (Savio *et al.*, 1990; Kastelic and Ginther, 1991). The increase in concentrations of IGFBP-4 and -5 in the follicular fluid of dominant follicles appears to precede this loss of functional dominance.

In conclusion, differences in follicle size and granulosa cell capacity to synthesize oestradiol were evident between potential dominant and subordinate follicles from as early as day 1 after ovulation, and preceded significant differences in granulosa cell response to LH and follicular fluid

concentrations of oestradiol, progesterone or IGFBP-4 and -5. Aromatase activity remained significantly higher in the largest follicle than in smaller follicles on all days except for day 2 after ovulation. No appreciable cAMP response to LH was evident in any follicle on day 1 after ovulation. However, by day 2, granulosa cell response to LH was greater in the largest follicle than in smaller follicles. Selection of the dominant follicle during the first follicle wave in cows is associated with increased granulosa cell aromatase activity followed by increased cAMP response to LH and follicular fluid oestradiol concentrations, and decreased follicular fluid concentrations of IGFBP-4 and -5 within 2 days after ovulation.

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