Development of preovulatory follicles expected to form short-lived corpora lutea in beef cows*

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Summary. Oestrus, expected to be followed by a short luteal phase, was induced in post-partum cows by weaning their calves at 35 days after parturition. Ovaries containing the first preovulatory follicles (Type F) formed after parturition were collected 3 h after the onset of oestrus. For comparison, preovulatory follicles (Type C) were collected 3 h after the onset of oestrus in normally cycling cows. The number of granulosa cells was determined and the concentrations of receptors for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in granulosa cells and for LH in theca cells were measured. Concentrations of oestradiol-17β, testosterone, androstenedione and progesterone in follicular fluid were also measured. Type F follicles contained about twice the number of granulosa cells (based on DNA) as did Type C follicles (45.8 ± 11.3 and 24.5 ± 3.9 μg DNA/follicle, respectively; P < 0.05) but these cells had fewer receptors for LH (0.13 ± 0.02 vs 0.29 ± 0.03 fmol/μg DNA; P < 0.01) and FSH (0.61 ± 0.08 vs 1.3 ± 0.29 fmol/μg DNA; P < 0.08) than did those from Type C follicles. Additionally, there were fewer receptors for LH in theca tissue from Type F than from Type C follicles (28.3 ± 5.2 vs 51.3 ± 6.1 fmol/follicle; P < 0.01). Concentrations of oestradiol-17β (475.8 ± 85.6 vs 112.9 ± 40.0 ng/ml; P < 0.01) and androstenedione (214.1 ± 48.7 vs 24.7 ± 7.7 ng/ml; P < 0.01) in follicular fluid were higher in Type C than in Type F follicles. There were therefore more granulosa cells in Type F follicles which contained fewer receptors for LH and FSH and accumulated less oestradiol-17β in the follicular fluid. We suggest that the characteristics of preovulatory follicles may influence the life-span of the subsequently formed corpus luteum and short luteal phases may be due, in part, to altered follicular development.

Keywords: cow; post partum; anoestrus; follicle; gonadotrophin receptors

Introduction

After extended periods of reproductive quiescence, the first ovulation is frequently followed by a luteal phase that is shorter in length than subsequent luteal phases. Short-lived corpora lutea are observed after the first ovulation after parturition in cows (Lamming et al., 1981; Humphrey et al., 1983) and sheep (Wise et al., 1986) and after the first ovulation of the breeding season in sheep (Yuthasakroskol et al., 1975; Walton et al., 1977). Additionally, the first ovulation at puberty in heifers (Gonzalez-Padilla et al., 1975), ewes (Berardinelli et al., 1980) and primates (Young & Yerkes, 1943; Wentz, 1979; Resko et al., 1982) is frequently followed by a short luteal phase.

It has been suggested that a short-lived corpus luteum is at least partly due to altered follicular development. Progestagen pretreatment of anoestrous cows induced to ovulate with exogenous gonadotrophin-releasing hormone (GnRH) or human chorionic gonadotrophin (hCG) resulted in

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higher serum concentrations of oestradiol-17β during the follicular phase than in cows not pre-treated with a progestagen (Sheffel et al., 1982; Garcia-Winder et al., 1986). Lower circulating concentrations of follicle-stimulating hormone (FSH) have been observed during the follicular phase in cows (Ramirez-Godinez et al., 1982a) and primates (Stouffer & Hodgen, 1980) preceding the formation of a short-lived corpus luteum. Garcia-Winder et al. (1987) found lower concentrations of oestradiol-17β in fluid from preovulatory follicles which would have resulted in short-lived corpora lutea. Hunter et al. (1986) and Inskeep et al. (1988) reported that developing follicles expected to form short-lived corpora lutea had fewer receptors for luteinizing hormone (LH) than did follicles expected to result in a luteal phase of normal length in ewes and cows. However, these studies utilized follicles that probably would not have ovulated if they had not been treated with exogenous gonadotrophins. Therefore, the following study was done to compare the number of receptors for LH and FSH and steroid concentrations in fluid of the first preovulatory follicles formed after parturition with preovulatory follicles in regularly cycling beef cows.

Materials and Methods

Animals. A group of 2-year-old crossbred beef cows that calved in the spring had their calves weaned about 35 days after parturition. Jugular blood samples were obtained daily from each cow for 10 days before calf removal and assayed for serum concentrations of progesterone (Niswender, 1973). Beginning at calf removal, cows were checked for oestrus every 2 h. The onset of oestrus was defined as the first observation time when the cow stood to be mounted. At 3 h after the onset of oestrus, local anaesthetic (lidocaine hydrochloride) was administered to each cow and the ovary containing the largest follicle was removed through an incision in the left paralumbar fossa. Follicles obtained from this group of animals were therefore the first preovulatory follicles formed after parturition and are referred to as Type F follicles (n = 7). Several studies have shown that this ovulation is associated with a short period of oestrus and is frequently followed by a short luteal phase (Odde et al., 1980; Ramirez-Godinez et al., 1981, 1982a, b). Preovulatory follicles were also obtained, 3 h after the onset of oestrus, from a group of crossbred beef cows that were cycling normally, and are referred to as Type C (control) follicles (n = 8).

Tissue processing. Ovaries were immediately placed on ice and transported to the laboratory where the largest follicle was carefully dissected from the surrounding ovarian stroma, frozen on solid CO₂, and stored in liquid nitrogen until assayed for gonadotrophin receptors (3–6 months). On the day that receptor assays were to be performed, whole follicles were removed from the liquid nitrogen and the average diameter of each follicle was determined. The follicles were placed at room temperature and allowed to begin thawing. After the theca had thawed but before the follicular fluid began to thaw, a cut was made in the theca. Because of the differential thaw rate of theca compared to the follicular fluid, the theca could be separated from the frozen follicular fluid containing the granulosa cells by a technique similar to that used by Murdoch et al. (1981) as modified by Braden et al. (1986). The diameter of the frozen follicular fluid was determined, to calculate the volume, and it was diluted to 4 ml with phosphate-buffered saline (0-01 M-NaPO₄; 0-14 M-NaCl; 0-22 mm-merthiolate; 0-1% (w/v) gelatin; pH 7-0). Granulosa cells were removed from the follicular fluid by centrifugation at 30 000 g for 15 min at 4°C. The diluted follicular fluid was aspirated and stored at −20°C until assayed for the concentration of steroids (oestradiol-17β, testosterone, androstenedione and progesterone). The pellet containing the granulosa cells was resuspended in 2 ml homogenization buffer (0-73 M-sucrose, 1 mm-EDTA; 25 mm-Tris; pH 7-4) and homogenized with 25 strokes in a Thomas 2AA30 glass homogenizer (Philadelphia, Pennsylvania, USA). Then 200 μl (10%) of the homogenate were removed and added to 200 μl phosphate-buffered saline containing EDTA (0-01 M-NaPO₄; 0-14 M-NaCl; 0-22 mm-merthiolate; 0-05 M-EDTA; pH 7-0). This sample was stored at −20°C until analysed for the content of DNA (Labarca & Paigen, 1980). The remaining granulosa tissue was centrifuged at 30 000 g for 15 min at 4°C in a preweighed tube. After centrifugation, the supernatant was decanted and the mass of granulosa membranes determined. The thecal layer was placed in homogenization buffer and homogenized for 10 sec in a polytron tissue homogenizer followed by 15 strokes in a Pyrex 15 ml ground-glass homogenizer. The homogenate was centrifuged at 30 000 g for 15 min at 4°C in a preweighed tube, the supernatant decanted and the mass of tissue determined.

Each tissue type was resuspended with assay buffer (10 mm-Tris; 1 mm-CaCl₂; 0-1% (w/v) bovine serum albumin; pH 7-4) to concentrations suitable for determination of receptor content (5 mg/ml for granulosa membranes and 25 mg/ml for the theca membranes).

Receptor assays. The numbers of receptors for LH and FSH were quantified in the granulosa membrane preparation and for LH in the thecal preparation by a standard curve technique (Nett et al., 1981; Braden et al., 1986). This technique involves the generation of a standard curve by incubating different quantities of a standard membrane preparation, of known receptor concentration as determined by Scatchard analysis (Scatchard, 1949), with a constant amount of radiolabelled hormone. In addition, samples of unknown receptor concentration were incubated with the same concentration of radiolabelled hormone. The concentration of receptors for the specific hormone in the sample
tissue was determined by direct comparison to the standard curve. This method has been shown to provide information similar to Scatchard analysis and has been validated for the quantification of receptors for LH, FSH (Braden et al., 1986) and GnRH (Nett et al., 1981). Granulosa tissue levels ranging from 0·1 to 30 mg (16 levels in triplicate) contained in 800 µl were used to generate the standard curves for both LH and FSH. The quantity of tissue used for samples (each in duplicate) was 4 or 20 mg for granulosa and theca tissue, respectively, contained in 800 µl. The quantity of radiolabelled hormone included was 50 000 c.p.m. (18 fmol 125I-labelled hCG, 22 fmol 125I-labelled FSH) contained in 100 µl. The specific activity of radiolabelled hormones was 25 µCi/µg for 125I-labelled hCG and 22 µCi/µg for 125I-labelled FSH. The proportion of radiolabelled hormones that could be bound to excess receptors was 38% for 125I-labelled hCG and 25% for 125I-labelled FSH. Assay conditions consisted of incubation for 16 h at room temperature in a shaker (80 cycles/min).

After incubation, 3 ml assay buffer were added to each tube and hormone bound to receptor was separated from free hormone by centrifugation (30 000 g; 15 min; 4°C). The supernatant was decanted and the quantity of hormone associated with the membrane pellet was measured using an Isodata 20/20 gamma counter. To evaluate non-specific binding of radiolabelled hormone, separate tubes were incubated with 400-fold excess unlabelled hormone (hCG or FSH) at each tissue level and for each sample tissue. Typically, non-specific binding was 2–3% of the total amount of radioactivity added. The limit of detection for LH and FSH receptors was 1 and 3 fmol, respectively. The intra-assay coefficients of variation were 2.2 and 2.3% for LH and FSH receptors, respectively.

Steroids in follicular fluid. Follicular fluid (10 µl) was diluted to 100 µl and extracted twice with 10 volumes of diethyl ether. The diethyl ether was decanted, dried under a stream of N2, and the steroids were resuspended in 0.5 ml hexane:benzene:methanol (85:15:5 by vol.). Steroids of interest were separated by elution from a Sephadex LH-20 column (0·5 × 17 cm) as described by Sluss (1981). After separation, androstenedione, progesterone, testosterone and oestradiol-17β were quantified by radioimmunoassay (Monkonpuna et al., 1975; Niswender, 1973; Berndtson et al., 1974; Thompson et al., 1979, respectively). The sensitivities of the radioimmunoassays were 25 pg, 12 pg, 10 pg and 4 pg for androstenedione, progesterone, testosterone and oestradiol-17β respectively. The intra-assay coefficients of variation were all <10%.

Statistics. All data were subjected to analysis of variance (Steele & Torrie, 1980). Data were transformed logarithmically when there was heterogeneity of variance. There was heterogeneity of variance in the number of receptors for FSH between groups which logarithmic transformation did not resolve. Therefore a t test not assuming equal variances was performed (Steele & Torrie, 1980).

Results

Before calf removal, none of the animals providing Type F follicles was cycling as indicated by serum concentrations of progesterone <0.5 ng/ml for 10 days before calf removal. Of 15 cows with calves removed, 11 (73%) showed oestrus between 2 and 8 days after calves were weaned. Three follicles ruptured during surgical collection and one cow had a luteinized follicle. These follicles were not included in any further analysis. Therefore, 7 follicles from cows expected to have short luteal phases and 8 follicles from cows expected to have luteal phases of normal length were utilized.

The mean (± s.e.) diameters of Type F follicles (17.6 ± 1.0 mm) were not different from those of Type C (16.8 ± 0.6 mm) (P > 0.1). The amount of DNA recovered from the granulosa layer of Type F follicles was about twice that recovered from Type C (45.8 ± 11.3 and 24.5 ± 3.9 µg/follicle, respectively; P < 0.05).

There was no difference for follicles of Types F and C in the total number of receptors in the granulosa layer for LH (5.5 ± 1.4 and 7.1 ± 1.7 fmol/follicle, respectively; P > 0.01) or FSH (26.1 ± 6.2 and 26.4 ± 3.8 fmol/follicle, respectively; P > 0.1). The concentration of receptors in the granulosa cells, expressed as fmol/µg DNA, was lower in follicles of Type F than of Type C for FSH (P < 0.08; Fig. 1) and LH (P < 0.01; Fig. 1). Additionally, the number of receptors for LH in theca, expressed as fmol/follicle, was lower in Type F than in Type C follicles (P < 0.01; Fig. 1). The concentrations of receptors for LH in theca are expressed as fmol/follicle because in the granulosa/theca separation procedure, any ovarian stroma adherent to the outside of the follicle after dissection cannot be separated from the theca, and this measure expressed per follicle removes any influence of different quantities of ovarian stroma between follicles or treatment groups.

The results for concentrations of steroids in follicular fluid are presented in Fig. 2. The concentration of oestradiol-17β was almost 4 times greater in Type C than in Type F follicles (P < 0.01). In addition, follicular fluid levels of androstenedione were higher in Type C than in Type F follicles.
Fig. 1. The concentrations of receptors for (a) LH and FSH in granulosa cells and (b) LH in thecal cells from follicles of Types F and C. Values are mean ± s.e. *P < 0.01 compared with value for Type C follicles.

Fig. 2. Concentrations of oestradiol-17β (E₂), testosterone (T), androstenedione (A) and progesterone (P₄) in follicular fluid from follicles of Types F and C. Values are mean ± s.e. *P < 0.01 compared with value for Type C follicles.

(P < 0.01). No difference was observed in the concentrations of testosterone or progesterone in fluid from follicles of Types F and C.

Discussion

The proportion of cows exhibiting oestrus within 8 days of weaning in the present study (11 of 15; 73%) is similar to that previously reported (Odde et al., 1980; Ramirez-Godinez et al., 1981, 1982a, b). In those studies, >80% of anoestrous cows that showed oestrus within 10 days after weaning of the calves had a short luteal phase after the first oestrus. Therefore, we assume that the first oestrus expressed by cows in the present study would have been followed by a short luteal phase.
The observation that Type F follicles had approximately twice the amount of DNA and therefore presumably twice the number of granulosa cells compared to Type C follicles was unexpected as other studies have indicated that, in many cases of luteal-phase defects, there have been fewer than the normal number of granulosa cells. Monkeys treated with pig follicular fluid had fewer granulosa cells than did untreated animals and developed short luteal-phase defects (Channing et al., 1979; Stouffer & Hodgen, 1980). In addition, Kreitmann et al. (1981) and Marut et al. (1983) have shown that, when granulosa cells were removed from preovulatory follicles of monkeys, the resulting corpora lutea had a normal life-span but did not secrete as much progesterone as normal corpora lutea. Oestradiol-17β and FSH are known mitogens for granulosa cells although in the present study the concentrations of oestradiol-17β in the follicular fluid were lower in Type F than in Type C follicles. Several possibilities could explain this observation. Follicles in the present study were collected 3 h after the onset of oestrus. Type F preovulatory follicles had a lower concentration of oestradiol-17β than did those of Type C. Since oestrous behaviour is dependent upon achieving critical circulating concentrations of oestradiol-17β, Type F follicles may have taken longer to synthesize enough oestradiol-17β for the expression of oestrus and so allowed more mitotic divisions of granulosa cells. The low concentration of receptors for LH may also have increased the time required for granulosa cells to synthesize sufficient oestradiol-17β for expression of oestrus. Another possibility is that granulosa cells from Type F follicles had completed one more ‘S’ phase (DNA synthesis) of the cell cycle than those from Type C follicles, and therefore would have twice the quantity of DNA per granulosa cell. We cannot distinguish between these possibilities in the present study as we did not directly quantify the number of granulosa cells. However, the most logical explanation is that Type F follicles have more granulosa cells than do those of Type C.

Several studies have suggested that granulosa cells in the preovulatory follicle form large (Type II) luteal cells (Donaldson & Hansel, 1965; McClellan et al., 1975; Alila & Hansel, 1984). A consequence of more granulosa cells contributing to the corpus luteum may be a greater proportion of large luteal cells in short-lived corpora lutea. Large luteal cells from sheep corpora lutea contain the majority of luteal receptors for prostaglandin (PG) F-2α (Fitz et al., 1982), and this could result in an enhanced sensitivity of the short-lived corpus luteum to the luteolytic effect of PGF-2α. This sequence of events is consistent with the observations of several groups. Roles for the uterus (Schirar & Martinet, 1982; Copelin et al., 1987) and prostaglandin (Troxl & Kesler, 1984a, b) in regression of short-lived corpora lutea have been implicated. However, Manns et al. (1983) measured peripheral serum concentrations of 15-keto-13,14-dihydro-PGF-2α as an indicator of PGF-2α release and did not observe any pulses of this metabolite during regression of short-lived corpora lutea that would be expected to occur during normal luteal regression. These data can be interpreted to indicate that short-lived corpora lutea are more sensitive to PGF-2α and undergo regression in response to levels of PGF-2α that do not cause regression of normal corpora lutea. This might be expected if there were more large luteal cells and thus more receptors for PGF-2α in short-lived corpora lutea. This possibility is not supported by the data of O’Shea et al. (1984) who did not show altered proportions of large luteal cells in corpora lutea expected to be short-lived. These corpora lutea were induced in anoestrous ewes by injection of GnRH which, as previously discussed, may not be comparable to the data presented here.

In the present study, granulosa cells from Type F follicles had fewer receptors for LH than did those from Type C follicles. To achieve the normal number of receptors for LH in granulosa cells, there must be adequate circulating concentrations of FSH and oestradiol. Additionally, granulosa cells must be able to respond to these hormones. In the present study, there tended to be fewer receptors for FSH on granulosa cells in follicles obtained from Type F follicles. Several investigators have reported lower circulating concentrations of FSH during the follicular phase preceding a short luteal phase (Wilks et al., 1976; Stouffer & Hodgen, 1980; Ramirez-Godínez et al., 1982a; García-Winder et al., 1986) when compared to the follicular phase of a normal length cycle. Additionally, the concentration of oestradiol-17β in follicular fluid of Type F follicles was ~25%
that in Type C follicles. While the number of receptors for oestradiol-17β was not determined in the present study, granulosa cells from Type F follicles tended to have fewer receptors for FSH which could limit the ability of these cells to respond to FSH. A lack of stimulation by oestradiol and FSH during the final stages of follicular development could result in fewer receptors for LH in granulosa cells of Type F follicles.

In the present experiment, we cannot distinguish whether the number of receptors for LH is limited by low levels of oestradiol-17β or whether concentrations of oestradiol-17β are reduced due to fewer receptors for LH. It is apparent that, after parturition, circulating concentrations and the frequency of pulses of LH are low compared to those in subsequent oestrous cycles (Humphrey et al., 1983). This could contribute to the low oestradiol-17β and androstenedione values in follicular fluid. Lower concentrations of androstenedione in follicular fluid of Type F follicles could reflect lower circulating values of LH or a lack of responsiveness of the thecal cells to LH. The similar levels of testosterone in the follicular fluid probably reflect 17-ketosteroid reductase activity in granulosa cells (Armstrong et al., 1981). In rat granulosa cells, this enzyme is not regulated by gonadotrophins (Bogovich & Richards, 1984), and so the greater number of granulosa cells in Type F follicles may give these follicles a greater capacity to convert androstenedione to testosterone. Therefore, lower levels of androstenedione in the follicular fluid of Type F follicles would be expected.

In summary, granulosa and theca cells from the first preovulatory follicle formed after calf removal in anoestrous beef cows have fewer receptors for gonadotrophins and accumulated less oestradiol-17β even though there are more granulosa cells than in subsequent preovulatory follicles. These differences could lead to the formation of a corpus luteum which is less responsive to gonadotrophins but more sensitive to PGF-2α, resulting in early regression and a short luteal phase.

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References


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