Gonadotropin requirements for dominant follicle selection in GnRH agonist-treated cows

J H Hampton, J F Bader, W R Lamberson, M F Smith, R S Youngquist¹ and H A Garverick

Department of Animal Sciences, 163 Animal Science Research Center, University of Missouri, Columbia, Missouri 65211, USA and ¹Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, Missouri 65211, USA

Correspondence should be addressed to H A Garverick; Email: garvericka@missouri.edu

Abstract

A study was conducted to examine the effects of gonadotropins on ovarian follicular development and differentiation in GnRH agonist (GnRHa)-treated cattle. Holstein cows were allotted into two pre-treatment groups: controls ($n = 5$) and GnRHa-treated ($n = 9$). Ovaries were removed from control cows on day 5 following a synchronized estrus. Treatment with GnRHa resulted in follicular arrest at <5 mm. Following follicular arrest, GnRHa-treated cows received a constant infusion of FSH for 96 h (GnRHa/FSH), with a randomly selected subset receiving hourly pulses of LH in addition to FSH during the last 48 h of infusion (GnRHa/FSH + LH). At the end of infusion, ovaries were removed, follicles were counted and measured, and follicular fluid samples were collected from large follicles (>10 mm). Differences in expression of mRNA for LH receptor, FSH receptor, cytochrome P450 side-chain cleavage, 3β -hydroxysteroid dehydrogenase, cytochrome P450 17α -hydroxylase (P450c17) and cytochrome P450 aromatase were determined in large follicles using *in situ* hybridization. The number of large follicles did not differ between GnRHa/FSH-treated and GnRHa/FSH + LH-treated cows ($P = 0.64$), but was greater than control animals ($P \leq 0.004$). Follicular fluid concentrations of estradiol- 17β and androstenedione were highest in GnRHa/FSH + LH-treated cows ($P \leq 0.04$), intermediate in control cows, and lowest in GnRHa/FSH-treated cows. Hybridization intensity of P450c17 was greater in GnRHa/FSH + LH-treated versus control or GnRHa/FSH-treated cows ($P \leq 0.03$). These results indicate that while FSH can support bovine follicular growth >10 mm, LH increases androgen production and expression of P450c17.

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Introduction

Ovarian follicular development during the bovine estrous cycle is characterized by two to three waves of follicular growth. Each wave is initiated by a transient increase in follicle-stimulating hormone (FSH) that stimulates a cohort of small antral follicles (2 to 4 mm in diameter) to continue to grow above 5 mm in diameter (Adams *et al.* 1992). During this time, expression of mRNA for cytochrome P450 side-chain cleavage and cytochrome P450 aromatase are induced in granulosa cells of the recruited follicles (Xu *et al.* 1995b, Bao *et al.* 1997a) coincident with the time that bovine follicles begin to produce estradiol- 17β (Skyer *et al.* 1987).

When the recruited pool of growing follicles reaches 8 to 9 mm in diameter, one follicle rapidly increases in size and becomes dominant over the remaining follicles that regress (Webb *et al.* 1999, Ginther 2000, Fortune *et al.* 2001). This divergence in size is accompanied by increases in estradiol- 17β production by the emerging dominant follicle

(Evans & Fortune 1997, Ginther *et al.* 1997). Previous studies in our laboratory have shown that, around the time of selection, expression of mRNA for luteinizing hormone (LH) receptor (LHr) and the steroidogenic enzyme, 3β -hydroxysteroid dehydrogenase (3β -HSD), is initiated in granulosa cells of dominant follicles (Xu *et al.* 1995a, Bao *et al.* 1997a,b). This time period coincides with the time when peripheral FSH concentrations reach a nadir (Adams *et al.* 1992), supporting the hypothesis that dominant follicle selection is associated with a shift from FSH to LH dependence. Administration of a gonadotropin releasing hormone (GnRH) agonist that suppressed circulating concentrations of LH, but not FSH, resulted in follicular arrest at 7 to 9 mm in diameter (Gong *et al.* 1996). The preceding data suggest that LH is important for continued follicular development. Consequently, we hypothesize that selection of a dominant follicle from a cohort of growing follicles may be dependent upon LH receptor expression in granulosa cells of the selected follicle.

The general aim of the current experiments was to examine the gonadotropin requirements for development of bovine follicles greater than 10 mm in diameter. To achieve this goal, we utilized the GnRH agonist (GnRHa) model developed by Gong *et al.* (1996). Previous studies (Gong *et al.* 1996, Garverick *et al.* 2002) have shown that this treatment results in suppression of the gonadotropins, FSH and LH, which subsequently results in suppression of follicular growth to <5 mm in diameter. Using this model, our specific aims were: (1) to determine if LH is required for continued follicular growth, (2) to examine the effects of exogenous FSH and LH on steroidogenesis via follicular fluid steroid content and follicular expression of steroidogenic enzymes, and (3) to determine if expression of LHR mRNA by granulosa cells is temporally or hormonally regulated.

Materials and Methods

Animals and treatments

This study was conducted under an approval granted by the University of Missouri Institutional Animal Care and Use Committee in accordance with all federal, state, and local regulations. Estrous cycles of 14 non-lactating Holstein cows that were >40 days postpartum were synchronized using a standard Ov-Synch protocol (Pursley *et al.* 1998). Briefly, cows received an i.m. injection of 100 µg GnRH (Cystorelin, Rhone Merieux, Athens, GA, USA), an i.m. injection of 25 mg prostaglandin (PG) F_{2α} (Lutalyse, The Upjohn Company, Kalamazoo, MI, USA) 7 days later, and an i.m. injection of 100 µg GnRH 48 h following PGF_{2α} injection. Ovarian structures were monitored daily by ultrasonography to determine time of ovulation and subsequent emergence of the first follicular wave. Five cows served as untreated controls and ovaries were removed via a flank incision within 24 h following detection of the dominant follicle (approximately 5 days following estrus). Dominant follicles were identified by daily ultrasonography beginning 24 h following estrus and were defined as a single antral follicle greater than 10 mm in diameter that emerged from a new cohort of recruited antral follicles. Five days following the final GnRH injection of the Ov-Synch protocol, the remaining cows (*n* = 9) were implanted s.c. with a single osmotic mini-pump (Alzet, model 2ML4; DURECT Corporation, Cupertino, CA, USA) containing 2.0 ml of the GnRH agonist, busarelin (Suprefact, Hoechst Marion Roussel, Uxbridge, UK; release rate = 2.5 µg/h). Prior to insertion, mini-pumps were incubated overnight in sterile saline at 37°C. Mini-pumps were implanted s.c. in the shoulder region under aseptic conditions and local anesthesia (7 ml lidocaine). Pumps remained *in situ* for 28 days and were replaced with a second osmotic mini-pump containing 2 ml busarelin, as described above, for an additional 28 days.

Following insertion of the second mini-pump, ovaries were examined daily using ultrasonography to detect

cessation of follicular growth. Following follicular arrest at <5 mm, cows (*n* = 9) were fitted with two indwelling jugular cannulae (one cannula/side) and received a continuous infusion of purified ovine (o) FSH (Ovagen; ICP Ltd, Auckland, New Zealand; 100 µg/h; equivalent to 2 units NIH-FSH-S1/h). In preliminary experiments, this concentration of FSH was required to induce follicular growth in GnRHa-treated cows (J H Hampton & H A Garverick, unpublished results). Forty-eight hours following initiation of FSH infusion, a group of the GnRHa-treated cows (GnRHa/FSH + LH; *n* = 4) were randomly selected to receive hourly pulses of purified bovine LH via the remaining jugular cannula (25 µg/h; USDA-bLH-B-6; 2.1 S1 units/mg) in addition to the continuous infusion of FSH (100 µg/h). Continuous infusion of FSH (100 µg/h) was maintained in the remaining GnRHa-treated cows (GnRHa/FSH; *n* = 5). All infusions were administered using two computer-controlled syringe pumps programmed for continuous or hourly pulses of FSH or LH respectively. Ovaries were monitored daily by ultrasonography until ovaries were removed from all GnRHa-treated cows via a flank incision 96 h after initiation of FSH infusion.

Tissue processing

Following removal, ovaries were immediately placed in sterile saline and cooled on ice. Size and number of surface follicles were determined prior to manual dissection. Follicles greater than 10 mm in diameter were dissected, measured and frozen over liquid nitrogen within 30 min of removal and stored at −80°C until sectioned. During freezing, 10 to 20 µl follicular fluid were aspirated using a tuberculin syringe and stored at −20°C until concentrations of progesterone (P₄), Δ⁴-androstenedione (A₄) and estradiol-17β (E₂) were determined by radioimmunoassay (RIA).

Blood collection and hormone analysis

Blood samples were collected daily via coccygeal venipuncture from the initiation of the study until ovaries were removed to assess peripheral concentrations of LH, FSH, and P₄. Blood samples were also collected every 12 min for 4 h, beginning 50 h following initiation of FSH infusion in GnRHa-treated cows, to verify delivery of and to characterize circulating concentrations of LH. Repeated samples were collected from the unused jugular cannula in GnRHa/FSH-treated cows, while in GnRHa/FSH + LH-treated cows the jugular cannula used for FSH infusion was employed for repeated sample collection. This cannula was aspirated prior to use to remove residual FSH. During the intensive sampling period, continuous FSH infusion was suspended in all GnRHa-treated cows and was replaced by hourly bolus doses (100 µg/h) of FSH. Repeated blood samples were also collected from control animals every 12 min for 4 h, 24 h prior to ovariectomy. Blood samples were allowed to clot overnight at 4°C and

centrifuged 24 h later at $1800 \times g$ for 30 min at 4°C. Serum was harvested and stored at -20°C until concentrations of P_4 , LH, and FSH were determined by RIA.

Serum concentrations of P_4 were determined using a direct, solid-phase RIA (COAT-A-COUNT; Diagnostic Products Corp., Los Angeles, CA, USA; Kirby *et al.* 1997) and daily samples were measured in duplicate 100- μ l aliquots of serum in a single assay, with an intra-assay coefficient of variation of 1.4%. Serum concentrations of LH were determined as previously described by Zaid *et al.* (1980) using anti-oLH TEA # 35 (J J Reeves, Washington State Univ., Pullman, WA, USA). Concentrations of LH were measured in duplicate 200- μ l aliquots of serum. Intra- and interassay coefficients of variation for three serum pools were 5.2 and 10.8% respectively, across seven assays. Serum concentrations of FSH were determined as previously described (Garverick *et al.* 1988). Concentrations of FSH were measured in duplicate 200- μ l aliquots of serum and NIAMDD oFSH RP-1 was used in reference preparations. Intra- and interassay coefficients of variation for three serum pools were 3.2 and 5.5% respectively, across two assays.

Concentrations of P_4 , A_4 , and E_2 in follicular fluid were determined using a direct, solid-phase RIA (COAT-A-COUNT; Diagnostic Products Corp.; Calder *et al.* 2001) after dilution (1:10 to 1:1000) in PBS. Dilution curves for samples of bovine follicular fluid were parallel to the standard curves of P_4 , A_4 , and E_2 assays. In the single P_4 , A_4 , and E_2 assays, intra-assay coefficients of variation were 1.8, 5.3, and 3.2% respectively.

In situ hybridization and image analysis

Expression of LH receptor (LHr) and FSH receptor (FSHr) mRNA in theca and granulosa cells was determined using *in situ* hybridization procedures previously described by our laboratory (Xu *et al.* 1995a, Bao *et al.* 1997a). In addition, localization of mRNAs encoding the steroidogenic enzymes, cytochrome P450 side-chain cleavage (P450scc), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17 α -hydroxylase (P450c17), and cytochrome P450 aromatase (P450arom) were examined (Xu *et al.* 1995b, Bao *et al.* 1997a,b). Briefly, sections of follicular tissue (14 μ m thickness) were prepared in a cryostat (-22°C) and mounted onto pre-chilled microscope slides. For each section, two slides were incubated with the antisense cRNA probe while one slide was incubated with the sense cRNA probe overnight at 55°C. Slides were washed, dipped in emulsion (Kodak, NB-2) and developed 3 to 14 days later. All follicles were classified morphologically as healthy or atretic (Xu *et al.* 1995b); only follicles classified as healthy were considered in subsequent analyses. Specific hybridization was quantified using a computer image analysis system (Scion Image for Windows v4.02, Scion Corporation, Frederick, MD, USA) and was defined as the mean hybridization (percentage of pixels containing a silver grain within a

defined field; two fields per follicle per slide) of the antisense cRNA probe minus the mean hybridization of the sense cRNA probe.

Statistical analyses

Differences in the number of follicles >10 mm in diameter were determined by analysis of variance using the general linear models procedures of SAS (version 8.02; SAS Inst. Inc., Cary, NC, USA). Differences among treatment least squares means were determined using pairwise *t*-tests comparisons (Ott 1993).

Effects of treatment on daily P_4 , LH, and FSH concentrations as well as on LH and FSH concentrations during the intensive sampling period were determined by repeated measures analysis of variance using the mixed models procedures of SAS (Littell *et al.* 1998). Cow within treatment was used as the error term, with time serving as the repeated measure and compound symmetry used as the covariance structure. Differences between treatment least squares means were determined when treatment-by-time interactions were significant using pairwise *t*-tests comparisons (Ott 1993).

Both LH pulse frequency (pulses/4 h) and pulse amplitude (ng/pulse) during the intensive sampling period were determined for individual cows as previously described (Cook *et al.* 1991). The following criteria were used to define an LH pulse: (1) an increase above the preceding nadir greater than or equal to two standard deviations above the mean LH concentration during the intensive sampling period for each individual cow, (2) the ascending portion of the peak could not contain more than one value between the nadir and the peak value, and (3) the peak value must be followed by at least two consecutive declining hormone values. Effects of treatment on LH pulse frequency and pulse amplitude were determined by analysis of variance using the general linear models procedures of SAS. Differences among treatment least squares means were determined using pairwise *t*-test comparisons (Ott 1993).

Due to heterogeneous variances and failure to obtain convergence with mixed model procedures for heterogeneous variance, analysis of ranks was performed for follicular fluid steroid concentrations and follicular mRNA expression prior to mean separation. Effects of treatment on ranked values were determined by repeated measures analysis of variance using the mixed models procedures of SAS (Littell *et al.* 1998). Cow within treatment was used as the error term, with time serving as the repeated measure and compound symmetry used as the covariance structure. Differences between transformed treatment least squares means were determined when treatment effects were significant ($P \leq 0.05$) using pairwise *t*-tests comparisons (Ott 1993). Analysis of variance of P_4 , A_4 , and E_2 in follicular fluid, as well as mRNA expression values for LHr, FSHr, P450scc, 3 β -HSD, P450c17 and P450arom were determined using the general linear models procedures of

SAS. Follicular fluid steroid concentrations and follicular mRNA expression data are reported as least squares means in figures and text. Standard errors are not shown because analyses were conducted as ranks.

Results

Serum hormone concentrations

Mean FSH concentrations (based on twice daily samples) are depicted in Fig. 1. In control animals, FSH concentrations were 0.90 ± 0.16 ng/ml 24 h following ovulation, tended to increase ($P = 0.06$) to a peak concentration of 1.25 ± 0.15 ng/ml approximately 48 h following ovulation and decreased ($P = 0.03$) to basal levels of 0.87 ± 0.15 ng/ml prior to ovariectomy. In GnRHa-treated animals, FSH concentrations prior to hormone infusion were 0.77 ± 0.15 ng/ml and 0.85 ± 0.17 ng/ml in GnRHa/FSH-treated and GnRHa/FSH + LH-treated cows respectively, and did not differ between GnRHa treatments ($P = 0.74$). Within 24 h following FSH infusion, peripheral concentrations of FSH had increased ($P \leq 0.007$) to 1.39 ± 0.15 ng/ml and 1.45 ± 0.19 ng/ml in GnRHa/FSH-treated and GnRHa/FSH + LH-treated cows respectively. Peripheral FSH concentrations did not differ by time or

Figure 1 Mean serum FSH (top panel) and LH (lower panel) concentrations (least squares mean $\pm$ S.E.; ng/ml) based on twice daily samples beginning prior to hormone infusion (Day -4, AM) and ending prior to ovariectomy (Day 0, AM). Initiation of LH infusion (Day -2, AM) in GnRHa/FSH + LH-treated cows is indicated on the top panel.

treatment ($P \geq 0.10$) in cows treated with FSH (GnRHa/FSH and GnRHa/FSH + LH) during the infusion period. Infusion of exogenous FSH resulted in peripheral FSH concentrations in GnRHa-treated cows that were not different ($P \geq 0.32$) from peak FSH concentrations in control cows.

Mean LH concentrations (based on twice daily samples; Fig. 1) did not differ ($P \geq 0.10$) in control, GnRHa/FSH-treated and GnRHa/FSH + LH-treated cows prior to LH infusion. Within 12 h of LH infusion in GnRHa/FSH + LH-treated cows, mean LH concentrations had increased to 1.6 ± 0.2 ng/ml and were greater ($P = 0.02$) than in GnRHa/FSH-treated cows. Within 24 h of LH infusion, mean LH concentrations in GnRHa/FSH + LH-treated cows (2.4 ± 0.2 ng/ml) were greater ($P \leq 0.001$) than in either GnRHa/FSH-treated (0.9 ± 0.2 ng/ml) or control cows (1.0 ± 0.2 ng/ml) and remained elevated ($P \leq 0.001$) until ovariectomy. Mean LH concentrations did not differ ($P \geq 0.10$) between control and GnRHa/FSH-treated cows during the infusion period.

During the intensive sampling period, mean LH concentrations were similar ($P = 0.81$) in control and GnRHa/FSH-treated cows (0.97 ± 0.27 ng/ml and 0.88 ± 0.27 ng/ml respectively), but were greater ($P \leq 0.002$) in GnRHa/FSH + LH-treated cows (2.6 ± 0.30 ng/ml). The number of LH pulses during the intensive sampling period was not different ($P = 0.15$) between GnRHa/FSH + LH-treated (3.0 ± 0.4 pulses/4 h) and control (2.2 ± 0.4 pulses/4 h) cows while both were greater ($P \leq 0.001$) than in GnRHa/FSH-treated cows (0 ± 0.4 pulses/4 h). LH pulse amplitude was greater ($P = 0.03$) in GnRHa/FSH + LH-treated cows (2.7 ± 0.4 ng/ml) than in control cows (1.1 ± 0.3 ng/ml).

Daily mean P_4 concentrations were $\leq 0.1 \pm 0.1$ ng/ml in GnRHa-treated animals during the infusion period and did not change over time ($P \geq 0.25$). In control cows, mean P_4 concentrations were similar ($P \geq 0.10$) to GnRHa-treated cows until 24 h prior to ovariectomy. By ovariectomy, mean P_4 concentrations had increased to 0.9 ± 0.1 ng/ml in control animals, indicating the presence of a developing corpus luteum.

Follicular dynamics

In control animals, ovulation was followed by recruitment of several small follicles (5 to 9 mm) within 48 h. A single dominant follicle was detected by ultrasonography within 4 days following ovulation (data not shown) and was confirmed prior to ovariectomy on day 5. In addition, corpora lutea were present in the ovaries from all control animals.

Prior to exogenous hormone infusion, all GnRHa-treated cows lacked follicular structures ≥ 5 mm in diameter. Within 48 h following FSH infusion, all GnRHa-treated cows had resumed follicular development and multiple follicles 6 to 9 mm in diameter were observed by ultrasonography (data not shown). Follicles continued to grow in all GnRHa-treated animals during the final 48 h of

4hormone infusion, and at ovariectomy all GnRHa-treated cows had multiple follicular structures >10 mm. Based on measurements collected immediately prior to dissection, the number of large follicles (>10 mm) did not differ between GnRHa/FSH-treated and GnRHa/FSH + LH-treated cows (10.8 ± 1.8 and 12.0 ± 1.8 respectively; $P = 0.64$), but were greater than in control animals (1.6 ± 1.6 ; $P \leq 0.004$).

Follicular fluid steroid concentrations

Concentrations of E_2 , P_4 , and A_4 were examined in follicular fluid collected from follicles greater than 10 mm in diameter and the results are summarized in Fig. 2. While there was no treatment effect on P_4 concentrations ($P = 0.30$), treatment effects were significant for E_2 and A_4 ($P \leq 0.01$). Concentrations of P_4 in follicular fluid in control, GnRHa/FSH-treated, and GnRHa/FSH + LH-treated cows were 54.0 ng/ml, 41.4 ng/ml, and 64.8 ng/ml respectively. Follicular fluid concentrations of E_2 were greater ($P \leq 0.04$) in GnRHa/FSH + LH-treated (790.7 ng/ml) cows compared with control (402.6 ng/ml) or GnRHa/FSH-treated (259.0 ng/ml) cows. Follicular fluid concentrations of A_4 were greater ($P \leq 0.03$) in GnRHa/FSH + LH-treated (45.3 ng/ml) and control (14.5 ng/ml) cows compared with GnRHa/FSH-treated cows (1.9 ng/ml).

Expression of mRNA for gonadotropin receptors and steroidogenic enzymes

Gonadotropin receptors

Hybridization intensity for LHR and FSHr mRNA in follicles greater than 10 mm in diameter is depicted in Fig. 3. Expression of mRNA for LHR was localized to theca and granulosa cells in all treatment groups. While LHR mRNA expression in granulosa cells was non-detectable in follicles <10 mm in diameter (data not shown), LHR mRNA was expressed in granulosa cells in all follicles >10 mm in diameter. There was no effect of treatment on LHR expression in theca ($P = 0.47$) or granulosa ($P = 0.78$)

cells (Fig. 3). Expression of mRNA for FSHr was not affected by treatment ($P = 0.69$) and was localized to granulosa cells of all follicles >10 mm diameter.

Steroidogenic enzymes

Expression of mRNA for P450scc in follicles >10 mm in diameter was localized to theca and granulosa cells and hybridization intensities are summarized in Fig. 3. There was no effect of treatment on P450scc mRNA expression in theca ($P = 0.47$) or granulosa ($P = 0.99$) cells.

Expression of 3β -HSD mRNA in follicles >10 mm in diameter was localized to theca and granulosa cells, but was not affected by treatment in either cell type ($P \geq 0.17$; Fig. 4).

Expression of mRNA for P450c17 and P450arom in follicles >10 mm was localized to theca and granulosa cells respectively, and is summarized in Fig. 4. Hybridization intensity of P450c17 was greater in GnRHa/FSH + LH-treated (49.2%) compared with control (35.5%; $P = 0.03$) and GnRHa/FSH-treated (15.8%; $P < 0.001$) cows. In addition, P450c17 expression in theca cells was greater ($P = 0.004$) in control compared with GnRHa/FSH-treated cows (Fig. 4). Expression of P450arom mRNA in granulosa cells was not affected by treatment ($P = 0.60$).

Discussion

In the present study, inhibition of gonadotropin secretion was accomplished by using a GnRH agonist model (Gong *et al.* 1996) that arrested ovarian follicular growth to less than 5 mm in diameter. Consistent with previous studies (Gong *et al.* 1996, Garverick *et al.* 2002), treatment with GnRHa reduced FSH concentrations such that a transient increase in FSH was inhibited and thereby prevented follicular growth greater than 5 mm in diameter. In control cows, recruitment of follicles to grow larger than 5 mm in diameter was associated with a post-ovulatory increase in FSH concentrations, followed by a decrease prior to the emergence of a single dominant follicle. This pattern of

Figure 2 Follicular fluid concentrations of estradiol-17 β , progesterone, and androstenedione (least squares mean $\pm$ S.E.; ng/ml) from follicles >10 mm in diameter. ^{a, b} Within estradiol 17- β results, columns with different superscripts differ $P < 0.05$; ^{c, d} within progesterone results, columns with different superscripts differ $P < 0.05$; ^{x, y} within androstenedione results, columns with different superscripts differ $P < 0.05$.

Figure 3 Expression of mRNA for LH receptor (LHr) and FSH receptor (FSHr) in granulosa and theca cells from follicles >10 mm in diameter (top panel) and expression of cytochrome P450 side-chain cleavage (P450scc) mRNA in granulosa and theca cells from follicles >10 mm in diameter (lower panel).

FSH secretion is consistent with previous studies that have described transient increases in FSH concentrations preceding each follicular wave in cattle (Adams *et al.* 1992, Webb *et al.* 1999). Infusion of FSH in GnRHa-treated cows resulted in FSH concentrations that were similar to peak concentrations in control animals and were associated with resumption of follicular growth. However, in GnRHa-treated animals, FSH concentrations throughout the infusion period remained similar to peak concentrations in control animals.

Maintenance of peak FSH concentrations for 96 h in GnRHa-treated cows resulted in multiple follicles that developed to >10 mm in diameter in the absence or presence of LH. The ability of FSH alone to promote follicular growth to preovulatory sizes has been demonstrated in sheep that were treated with a GnRH antagonist and exogenous FSH for at least 3 days (Campbell *et al.* 1998, 1999). However, in studies conducted with GnRHa (Gong *et al.* 1996, Garverick *et al.* 2002) or GnRH-immunized heifers (Crowe *et al.* 2001), treatment with exogenous FSH for 48 h did not result in follicle growth greater than 9 mm in diameter. In GnRH-immunized heifers, continued treatment with exogenous FSH for six days resulted in the growth of multiple large follicles; however, the number of large follicles was nearly three times greater in GnRH-immunized heifers treated with both porcine (p) FSH and

Figure 4 Expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA in granulosa and theca cells from follicles >10 mm in diameter (top panel) and expression of cytochrome P450 17α-hydroxylase (P450c17) mRNA in theca cells and cytochrome P450 aromatase (P450arom) mRNA in granulosa cells from follicles >10 mm in diameter (lower panel). ^{a,b,c} Within P450c17 results, columns with different superscripts differ $P < 0.05$.

pLH (Crowe *et al.* 2001). Therefore, the ability of FSH to promote follicle growth >10 mm in the absence of pulsatile LH may be dependent on the length of FSH treatment. These data and those of Crowe *et al.* (2001) indicate that prolonged exposure to FSH alone can stimulate and support the growth of follicles >10 mm in diameter.

While follicle size and number were not dependent on LH concentrations in the current study, steroid content of follicles was affected by changes in serum LH concentrations. Based on samples collected from GnRHa/FSH-treated cows during the intensive sampling period, GnRHa-treatment abolished LH pulsatility and restricted LH concentrations to basal levels similar to previous studies (Gong *et al.* 1996). Treatment with exogenous LH restored LH pulse patterns that were of similar frequency, but of higher mean concentration and pulse amplitude, than control cows. These changes in LH pulsatility were associated with increases in follicular fluid steroid concentrations in GnRHa/FSH + LH-treated cows relative to GnRHa/FSH-treated cows. In GnRHa-treated cows, infusion of LH in a pulsatile pattern resulted in a 40-fold increase in follicular fluid A_4 concentrations as well as a threefold increase in follicular fluid E_2 concentrations. These results are similar to those described by Campbell

et al. (1998, 1999) and Crowe *et al.* (2001) in which LH stimulation was required for steroidogenesis in GnRH antagonist-treated ewes and GnRH-immunized heifers respectively.

However, coincident increases in follicular steroid concentrations and LH pulsatility were not associated with changes in expression of mRNA for P450scc in theca or granulosa cells or P450arom in granulosa cells since hybridization intensities of these steroidogenic enzymes were similar among all treatment groups. These results are similar to a previous study from our laboratory in which infusion of LH in a high pulse frequency and amplitude pattern during the luteal phase did not increase expression of P450scc or P450arom in either cell type (Manikkam *et al.* 2001). These data agree with previous reports from Tian *et al.* (1995) that showed no increase in P450arom expression following luteolysis in cattle, despite dramatic increases in follicular fluid concentrations of A₄ and E₂. However, unlike reports from our laboratory, increased expression of P450scc coincided with increases in LH concentrations (Tian *et al.* 1995). This disparity may be due to sensitivity differences in the methods used to quantify mRNA expression.

All follicles examined in the current experiment were exposed to similar concentrations of FSH during recruitment, but differences in LH pulsatility did not alter P450scc or P450arom mRNA expression in granulosa cells. Perhaps expression of these enzymes in granulosa cells may be more sensitive to FSH rather than to LH concentrations. Previous experiments from our laboratory have shown that expression of mRNA for P450scc and P450arom in granulosa cells are induced following the transient rise in FSH that precedes recruitment of follicles (Xu *et al.* 1995b, Bao *et al.* 1997a). In addition, Garverick *et al.* (2002) showed that infusion of FSH into GnRHa-treated heifers increased expression of mRNAs for P450scc and P450arom in granulosa cells. The preceding results in cattle are supported by the observation that granulosa expression of P450scc and P450arom is down-regulated in the ovaries of FSH β and FSHr knockout mice (Burns *et al.* 2001).

While not significantly ($P = 0.17$) different in the current experiment, expression of mRNA for 3 β -HSD was nearly two times higher in theca and granulosa cells of control and GnRHa/FSH + LH-treated cows, implying that perhaps LH pulses can increase expression of this steroidogenic enzyme in both cell types. Induction of 3 β -HSD mRNA expression in granulosa cells has been associated with follicular selection in cattle (Bao *et al.* 1997b), a period that coincides with decreasing concentrations of circulating FSH and increasing concentrations of LH (Kulick *et al.* 1999). In addition, Tian *et al.* (1995) have shown that expression of mRNA for 3 β -HSD increases in bovine theca and granulosa cells in preovulatory follicles following luteolysis, while Manikkam *et al.* (2001) reported increases in this enzyme in granulosa and theca cells from 2nd wave dominant follicles following infusion

with exogenous LH. Therefore, increases in LH concentrations may enhance 3 β -HSD expression in theca and granulosa cells.

LH pulsatility had the most marked effect on expression of mRNA for P450c17 in theca cells. Hybridization intensity of P450c17 was highest in GnRHa/FSH + LH-treated cows, intermediate in control cows, and lowest in GnRHa/FSH-treated cows, indicating that as LH concentrations increased, expression of mRNA for P450c17 increased accordingly. In addition, these effects of LH on expression of mRNA for P450c17 were mirrored in follicular fluid concentrations of A₄ and E₂. This indicates that of the steroidogenic enzymes examined in this study, P450c17 mRNA expression was the most sensitive indicator of androgen production by theca cells and of estrogen production by granulosa cells. Increases in P450c17 mRNA expression have also been observed in 2nd wave dominant follicles when heifers were infused with exogenous LH (Manikkam *et al.* 2001). Garverick *et al.* (2002) reported that increases in expression of mRNA for P450c17 were associated with increasing follicular size in follicles <10 mm in diameter. In addition, GnRHa-treated heifers that were infused with exogenous FSH had higher P450c17 expression than untreated controls and heifers treated with GnRHa only. In the current experiment, expression of mRNA for P450c17 was lowest in GnRHa/FSH-treated cows, despite higher FSH concentrations than controls. However, these findings do not necessarily contradict those found in the previous experiment since different sized follicles were examined in each experiment.

Despite treatment differences in pulsatile release of LH and follicular steroid concentrations, LHr mRNA expression in granulosa or theca cells was not affected by treatment. Our original hypothesis was that LHr expression in granulosa cells was hormonally controlled either directly by E₂ or indirectly through LH stimulation of thecal androgen production and would only be expressed in animals with adequate LH support. Previous studies had shown that induction of granulosa LHr in rodents was dependent upon both FSH and E₂ concentrations (Segaloff *et al.* 1990). Accordingly, in cattle, increases in E₂ concentrations preceded LHr mRNA expression in granulosa cells (Bodensteiner *et al.* 1996, Evans & Fortune 1997). Therefore, it was surprising that LHr mRNA expression was not reduced in GnRHa/FSH-treated cows despite a 36% decrease in E₂ concentrations versus control cows. However, E₂ concentrations were still detectable in GnRHa/FSH-treated cows in the absence of detectable LH pulses. Therefore, it is possible that basal LH concentrations provided adequate support for granulosa E₂ production which, in turn, was able to induce LHr expression in granulosa cells. Alternatively, it is possible that maintenance of high FSH concentrations alone may have induced expression of granulosa LHr and perhaps this was associated with the ability of FSH to override selection. Regardless, these data show that exposure to high FSH, but basal

LH, concentrations, in the presence of E₂ are sufficient to induce expression of LHr in granulosa cells.

In conclusion, the results from the present study indicate that FSH alone can support bovine follicular growth >10 mm. However, LH released in a pulsatile manner dramatically increases steroidogenic output from large, growing follicles. In addition, expression of mRNAs encoding P450c17 in theca cells appeared to be uniquely associated with circulating LH concentrations. However, expression of mRNA for LHr in granulosa cells was not associated with changes in LH pulsatility. These results provide further insight into the role of the gonadotropins in follicular development and differentiation.

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