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Hormonal Regulation of Thecal Cell Function during Antral Follicle Development in Bovine Ovaries*

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ABSTRACT. The hormonal regulation of thecal cell function was investigated with cells isolated at various stages of antral follicle development. Bovine thecal cells were isolated from small antral, medium antral, and large Graffian follicles (small, medium, and large ovarian follicles). Serum-free cultures of thecal cells were established and viable for a minimum of 6–8 days of culture. The purity of the thecal cell population was characterized cytochemically and was found to contain less than 5% endothelial cell and/or granulosa cell contamination. The steroidogenic capacity of this purified population of thecal cells in serum-free culture was examined through an analysis of androgen and progesterone production. Androgen production was high during the first 3 days of culture, then declined to undetectable levels. Production of androstenedione was approximately 10-fold higher than production of testosterone. Progesterone production remained relatively constant throughout the 8-day culture period. hCG was found to stimulate androgen production during days 1–3 of culture, but had a negligible effect on progesterone production. In contrast, hCG stimulated progesterone production during days 3–6 of culture, but had a negligible effect on androgen production. Insulin stimulated progesterone production during days 3–6 of culture, but had no effect on androgen or progesterone production during days 1–3 of culture. The minimum effective concentrations of hCG and insulin required to stimulate steroidogenesis of the thecal cells ranged from approximately 1–10 ng/ml. Addition of serum to the cultures decreased androgen production and suppressed the hormone responsiveness of the cells. Thecal cells in culture appear to alter their steroidogenic capacity from an androgen-producing cell to a progesterone-producing cell. Analysis of the developmental regulation of thecal cell function revealed that androgen production and hormone responsiveness were relatively constant in small, medium, and large follicles. In contrast, progesterone production and hormone responsiveness were highest in small follicles, intermediate in medium follicles, and lowest in large follicles. A more general analysis of the developmental regulation of thecal cell function examined the secretion of radiolabeled proteins. A large number of radiolabeled proteins were secreted by thecal cells, ranging in molecular mass from 5–500 kDa. Interestingly, insulin and hCG had no major effect on secretion of proteins by cells isolated from any of the stages of development examined. The major developmental change in radiolabeled secreted proteins was a decline in the abundant secretion of proteins greater than 100 kDa by thecal cells from small follicles as follicle development progressed. Combined results suggest the following. 1) Bovine thecal cells in culture appear to alter steroidogenic capacity, reflected in a switch in predominant secretion of androgen to progesterone. 2) hCG/LH has a major role in the stimulation of thecal cell steroidogenesis, and insulin at nearly physiological concentrations may regulate progesterone production. 3) As a small antral follicle develops into a large Graffian follicle the ability of the thecal cells to produce androgen remains relatively constant, while the ability to produce progesterone and several secreted proteins declines. The serum-free culture of bovine thecal cells is anticipated to be useful to elucidate the importance of thecal cells in the hormonal and developmental regulation of ovarian physiology. (*Endocrinology* **127:** 2907–2917, 1990)

THE OVARY is a dynamic organ due to the continual process of follicular development and atresia that occurs throughout the estrous cycle (1–4). An exceedingly complex regulation of cell growth and differentiation is necessary for ovarian physiology. The ovarian follicle develops through several distinct stages, defined as primordial, antral, and Graffian follicles. In addition, follicles may undergo atresia at any stage of development, and ovulated follicles may luteinize to form corpus luteum. Different follicles, however, are not necessarily predestined for a specific stage of development or atresia, since a number of observations have demonstrated that follicle development can be manipulated through various endocrine treatments (5–7) or electrocauterization (8). Although recent investigation of follicle development has been performed with molecular probes to the steroidogenic enzymes in the cells (9–11), the molecular and cellular events that occur during follicular development are not fully understood. This is especially true for the thecal cell component of the follicle.

The thecal cell has a critical role in the control and maintenance of ovarian function. Thecal cells provide structural integrity for the ovarian follicle and are in contact with the basal surface of the mural granulosa cells, allowing for cell-cell interactions between these two cell types. A critical steroid-mediated interaction between thecal and granulosa cells involves the ability of

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the thecal cells to synthesize androgens in response to LH which are then used by granulosa cells for aromatization to estrogen (12). In addition to steroid-mediated thecal-granulosa cell interactions (12, 13), thecal cells produce several nonsteroidal paracrine factors. Thecal cells have been shown to produce transforming growth factor-β (14) and transforming growth factor-α (15), which can influence the growth and differentiation of both granulosa cells (14–19) and thecal cells (14, 15). With the increasing awareness of the potential importance of interactions between thecal and granulosa cells, further elucidation of the hormonal and developmental regulation of thecal cell function is required.

Thecal cells have been obtained as tissue explants from the ovaries of a number of species (20–30), and thecal-interstitial cell preparations have been obtained from rat ovaries (31–34). Dispersed thecal cell cultures have been established for porcine (35–38) and domestic fowl (39, 40), which have a different steroidogenic capacity than theca from rat, bovine, or human. The small size of the rat ovary and the limited availability of human follicles make studies on purified dispersed thecal cells from these species difficult. The bovine is an ideal mammal for determining developmental and hormonal aspects of thecal cell function, in part due to the large size of the bovine ovary and the quantity of tissue available. In addition, the bovine is similar to the human in the endocrine control of ovarian function, and both species are considered monoovulatory. Therefore, factors controlling growth and differentiation may potentially be similar in the human and bovine. Although theca interna cell explant cultures have been studied (13, 21, 23), serum-free culture of dispersed bovine theca interna cells has not been used to investigate the hormonal and developmental regulation of thecal cell function. An analysis of serum-free cultures of bovine granulosa cells has provided insight into the regulation of granulosa cell function during follicle development (41). The current study was designed to establish a serum-free culture system for bovine theca interna cells isolated from small antral, medium antral, and Graffian follicles to investigate the developmental and hormonal regulation of thecal cell function.

Materials and Methods

Cell preparation and culture conditions

Bovine ovaries were obtained from young nonpregnant cycling heifers slaughtered at an abattoir and placed immediately on ice (Research Supply, Inc., Nashville, TN). Healthy developing follicles were identified and classified as previously described (41, 42) and dissected from the ovaries under sterile conditions. Healthy developing follicles were distinguished from late stage atretic follicles by the presence of a pink vascularized thecal cell layer and clear follicular fluid free of cell debris. Follicles were punctured with a hypodermic needle, and the follicular fluid was aspirated. Granulosa cells were removed by cutting the follicle in half, agitating the follicle in buffer, and gently scraping the follicle wall with a fine plastic loop in a beaker containing Ham's F-12 (Gibco, Grand Island, NY). The theca interna cell layer was microdissected away from the theca externa and cleaned of any adhering granulosa cells. The theca interna layers were minced into small pieces and digested for approximately 1 h at 37 C in Ham's F-12 containing 1 mg/ml pronase, 1 mg/ml collagenase, 1 mg/ml hyaluronidase, and 0.01 mg/ml DNase. Cell dispersion was facilitated by agitating the solution back and forth through a Pasteur pipette at 15- to 20-min intervals during the digestion. Dispersed cells were centrifuged for 4 min at 50 $\times$ g and resuspended in medium for plating.

Initial studies concerning the development of a serum-free culture used dispersed theca from all sizes of follicles. These cells were plated in a Ham's F-12 with or without 10% newborn calf serum in the presence or absence of hCG (1 μg/ml; Calbiochem, La Jolla, CA) plus insulin (5 μg/ml; Sigma, St. Louis, MO). Cells were plated in 1 ml medium in 24-well Linbro plates and cultured at 37 C in a 5% CO_2 atmosphere. Cell function was assessed by RIA of progesterone, androstenedione, and testosterone in medium collected from plates terminated on days 1, 2, 3, 6, and 8 of culture. The number of cells existing at the end of each culture period was assessed during culture by quantitating DNA levels. Unless otherwise designated, medium was collected and changed in cultures on days 3 and 6 when cultures exceeded 3 days in length. Hormone treatments were added to medium at plating and on days 3 and 6, when new medium was added to the cultures.

Studies undertaken to characterize functional differences and hormonal regulation of thecal cells at different stages of development were carried out in a fashion similar to that described above, except that thecal cells were obtained from small (<5 mm), medium (5–10 mm), and large (>10 mm) follicles and cultured separately. The exact experimental conditions and treatments are outlined in *Results*.

Cell morphology and purity

The cell morphology of cultures terminated on days 3 and 6 was determined with an inverted phase contrast microscope. Cells were fixed with 4% buffered formalin, stained with Gill hematoxylin (Fisher, Atlanta, GA), and counterstained with acidic eosin. Cell purity was determined cytochemically with the detection of high levels of cell surface low density lipoprotein (LDL) receptors with fluorescent-labeled LDL, as previously described (43). Endothelial cells and granulosa cells were found to stain positive for LDL, while thecal cells in the absence of hormones did not. Briefly, cells were plated on Thermanox plastic coverslips (Lux, Miles Scientific, Naperville, IL) for 24 h and incubated with 5 μg/ml fluorescent LDL (Biomedical Technologies, Stoughton, MA) for 4–6 h at 37 C in a 5% CO_2 atmosphere. Labeled cells were rinsed and fixed with buffered formalin, mounted on a glass slide, and examined with a fluorescent microscope. The number of positive stained cells was determined in a field of a minimum of 100 total cells, using 5 separate fields with an individual cell preparation.

Gel electrophoresis and fluorography

Protein synthesis by thecal cells at different stages of development was evaluated in cells cultured in glycine- and methionine-free medium treated with [^{35}S]methionine (5 μCi/ml) and [^{3}H]glycine (5 μCi/ml). Medium was collected after 48 h of culture, centrifuged, and electrophoresed on sodium dodecyl sulfate (SDS)-5–15% polyacrylamide gradient slab gels (44) under reducing conditions using the Laemmli (45) buffer system. Fluorography was performed, as described previously, with diphenyloxazole in acetic acid (46).

DNA assay

At the end of a culture period medium was removed from cells, centrifuged to remove cellular debris, and frozen at −20 C for storage until assayed. Cells were then sonicated in 0.5 ml ethidium bromide buffer/well (20 mM sodium chloride, 5 mM EDTA, and 10 mM Tris, pH 7.8), and DNA was determined fluorometrically with ethidium bromide (44) by incubating 100–200 μl sonicated cell suspension with 1 U heparin and 667 ng ethidium bromide in a volume of ethidium bromide buffer to bring the total volume to 1 ml. The incubation was carried out at room temperature for 30 min, and fluorescent emission was determined at 585 nm with 350 nm excitation. Quantification of DNA was determined by extrapolation from a standard curve containing calf thymus DNA. This assay has a sensitivity of approximately 0.1 μg DNA and is linear up to 3 μg DNA.

Steroid RIAs

Progesterone was quantitated by a previously validated RIA (47). Testosterone was quantitated using the Coat-A-Count total testosterone RIA kit purchased from Diagnostic Products Corp. (Los Angeles, CA). Androstenedione was quantitated by RIA using the AN6–22 antibody purchased from Endocrine Sciences RIA reagents (Tarzana, CA). Conditioned medium (1–200 μl) was incubated with 100 μl antibody diluted 1:50 with gelatin buffer [0.124% (wt/vol) gelatin, 0.15 M NaCl, 0.01 M Na_2 EDTA, and 0.05 M Tris, pH 7.5], 150 fmol 1β,2β-[^{3}H] androstenedione (New England Nuclear, Boston, MA) in 100 μl gelatin buffer, and a volume of Ham's F-12 medium to bring the final volume to 400 μl. This mixture was incubated at 4 C for 16–20 h, after which 0.25 ml gelatin buffer containing 0.05% dextran and 0.5% charcoal was added, incubated for 5 min at 4 C, and then centrifuged at 2600 × g for 15 min. The radioactivity in the supernatant was then determined. Androstenedione (Sigma, St. Louis, MO) was used as a reference in the assay. The sensitivity of the assay was 12.5 pg/ml. Intra- and interassay (n = 8) coefficients of variation were 4% and 11%, respectively. Levels of progesterone, testosterone, and androstenedione were undetectable in medium containing 10% calf serum.

Statistical analysis

Data from the time-course studies indicated that basal levels of steroid production fluctuated among experiments. Therefore, paired t tests were used to determine differences between the presence or absence of serum in culture medium and to determine if a combination of hCG and insulin increased steroidogenesis over that in nontreated control cultures. A paired t test was also used to compare hCG and insulin stimulation between cultures with and without serum and to determine differences between DNA contents in culture wells in cells cultured in the presence or absence of serum. Changes in DNA and accumulation of steroids over time were analyzed within treatment by analysis of variance using a protected least significant difference (LSD) procedure ($P < 0.05$) for pairwise comparisons. In studies designed to evaluate the effects of hCG, insulin, and serum on steroidogenesis by thecal cells from different sized follicles, data were analyzed within follicle size by analysis of variance for a randomized block design, using experimental replicate as the blocking factor, and a protected LSD procedure was used to make individual pairwise comparisons between treatments. When heterogeneous variances between the treatments existed, the square root of the data were used in these analyses. Differences between the steroidogenesis of thecal cells from different sized follicles were also analyzed by analysis of variance for a randomized block design, using experimental replicate as the blocking factor and a protected LSD procedure to make individual pairwise comparisons. Each study was replicated three to five times, with two or three observations per treatment within replicates. All data concerning the accumulation of steroids have been normalized to the DNA content in culture wells at the time of medium collection.

Results

The morphology of thecal cells cultured in the absence of serum is shown in Fig. 1. The data presented are representative of cells on day 3 or 6 of culture. A stromal or fibroblast-like morphology is primarily observed. Examination of high levels of LDL receptor was determined to investigate the purity of the thecal cell population. Fluorescent LDL binding was intense on endothelial cells, less intense on granulosa cells, and absent on thecal cells cultured in the absence of hormones. A low level of LDL binding was observed on thecal cells cultured in the presence of hormones (data not shown). These observations allowed the development of a procedure to assess the purity of the thecal cell preparation. The presence of fluorescent LDL binding to cells cultured in the absence of hormones was used to quantitate granulosa cell and/or endothelial cell contamination of the thecal cell cultures. The percentage of cell contamination in the thecal cell cultures was 4 ± 2%. These data were obtained with replicate determinations from four different thecal cell preparations. The absence of appreciable granulosa cells in the thecal cell preparation was also confirmed by the observation that aromatase activity was undetectable in the thecal cell cultures when quantitated as described previously (41). The DNA levels observed in serum-free cultures indicate that cell numbers declined ($P < 0.01$) between days 1 and 3 of culture and then remained relatively constant, whereas cells cultured in the presence

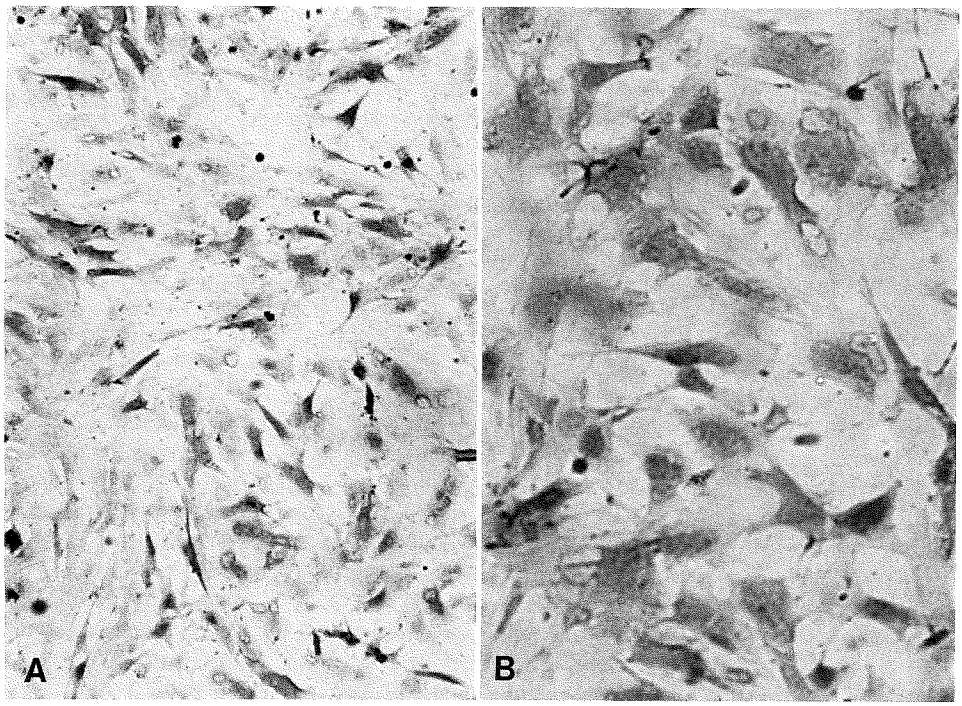

FIG. 1. Morphology of hematoxylin- and eosin-stained bovine thecal cells cultured in the absence of serum for 3 days and photographed at ×180 (A) and ×360 (B) magnification. This is a representative photograph from three different experiments with three different cell preparations.

of serum remained relatively constant throughout the 8 days of culture (Fig. 2). Addition of hCG and insulin to cells resulted in DNA levels that were slightly greater but not statistically significant than those in cells cultured without hCG and insulin. Potential fluctuation in cell number during culture indicated the importance of normalizing all data to DNA levels at the time of medium collection.

Analysis of androgen production by thecal cells was the initial cellular function investigated. Accumulation of testosterone (Fig. 3A) and androstenedione (Fig. 3B) in culture medium was greatest during the first 3 days of culture. Androgen production between days 3–6 of culture significantly decreased and was generally undetectable between days 6–8 of culture. Levels of androstenedione produced were approximately 10-fold higher than levels of testosterone. Accumulation of androstenedione and testosterone during days 0–3 of culture was greater ($P < 0.05$) in serum-free cultures than in cultures containing serum. Treatment with a combination of hCG and insulin increased ($P < 0.05$) accumulation of both androgens in the absence (Fig. 3) and presence of serum (data not shown). However, the quantity of androstenedione accumulated in medium of hCG- and insulin-treated cells cultured in the presence of serum (3.02 ± .27 ng/μg DNA·72 h) was lower ($P < 0.05$) than that in untreated serum-free cultures (4.76 ± 0.18 ng/μg DNA·72 h) during days 0–3. Therefore, androgen production by bovine thecal cell cultures declines after culture for 3 days, and cotreatment with hCG and insulin stimulates androgen production, while serum reduces androgen accumulation.

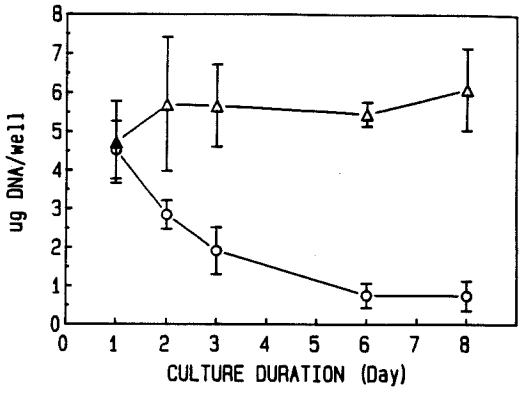

FIG. 2. Levels of DNA (micrograms per well) from bovine thecal cells cultured in the absence (○) or presence (△) of serum. Data are presented as micrograms of DNA per well. Values represent the mean ± SEM from three different experiments. Values on days 3, 6, and 8 differ ($P < 0.01$) from each other.

The characteristics of progesterone production by thecal cell cultures were different from those of androgen production (Fig. 3C). The basal level of progesterone production remained relatively constant throughout the 8 days of culture. Treatment with hCG and insulin had negligible effects during the first 3 days of culture, but significantly stimulated progesterone production between days 3–6 of culture. Serum increased the basal production of progesterone on day 2 of culture. Serum reduced ($P < 0.05$) the ability of hCG and insulin to stimulate progesterone accumulation between days 3–6 and 6–8 of culture (Fig. 3C). Therefore, while progesterone production was high in response to hormones be-

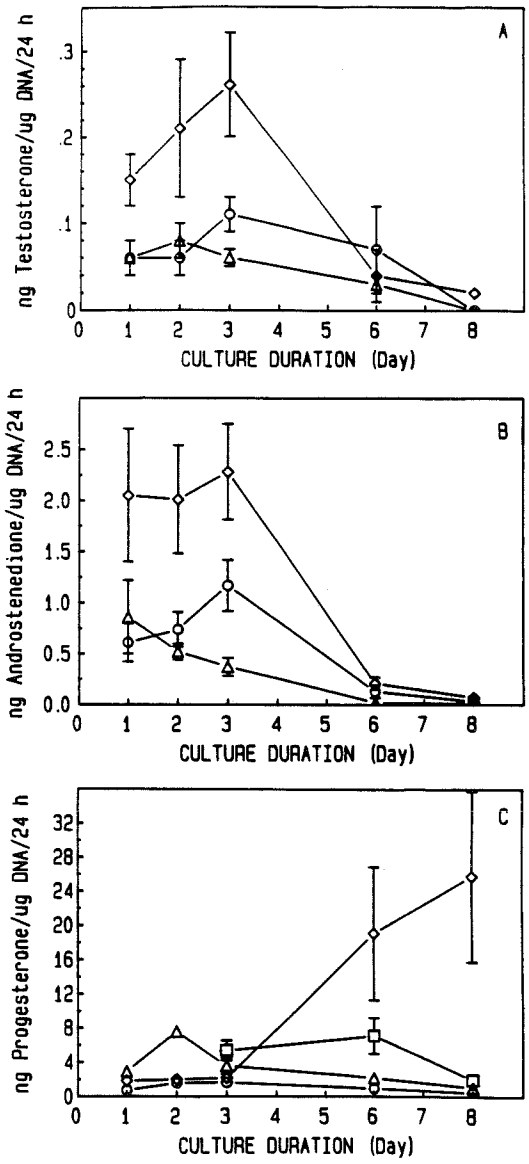

FIG. 3. Production of testosterone (A), androstenedione (B), and progesterone (C) in cultures of bovine thecal cells. Values represent nanograms of steroid accumulated, normalized for micrograms of DNA per well divided by the number of days of accumulation (nanograms per μg DNA/24-h period). Medium was collected on days 1, 2, 3, 6, and 8 of culture. Cells are cultured in the absence of serum and hormones (○), in the absence of serum and the presence of hCG and insulin (◇), in the presence of serum and the absence of hormones (△), and in the presence of serum, hCG, and insulin (□). Each *point* represents the mean ± SEM of three different experiments.

tween days 3–8 of culture, androgen production was low and nonresponsive. In contrast, androgen production was high and hormone responsive during the first 3 days of culture when progesterone production was nonresponsive to hormones.

Information obtained from the androgen and progesterone time-course studies was used to determine optimal periods to study hormone responsiveness in a developmental sense. The optimal time to measure steroid production was determined for androgens to be a 72-h accumulation in the first 3 days of culture, while progesterone production was measured in a 72-h collection between days 3–6 of culture. Androstenedione and progesterone production by thecal cells isolated from small, medium, and large follicles is shown in Figs. 4 and 5. Thecal cell production of androstenedione remained constant during the different stages of development. Androstenedione production was stimulated by hCG, but not by insulin. In addition, no apparent synergisms occurred with combined treatment of hCG and insulin. Androgen production increased approximately 2-fold in response to hCG and was similar for cells from all stages of development (Fig. 4). Cells from all stages of development produced significantly lower levels of androstenedione in the presence of serum. Progesterone production during the first 3 days of serum-free culture was similar for cells from small, medium, and large follicles, with no significant response to hCG and/or insulin (Fig. 5A). In contrast, progesterone production was dramatically enhanced ($P < 0.001$) in cells from small follicles, tended to be increased in cells from medium ($P < 0.07$) follicles, and was not altered in theca from large follicles when cultured in the presence of serum. Therefore, in the first 3 days of culture, thecal cells from small, medium, and large follicles produced androgen in response to hCG, while progesterone production was not responsive to hCG or insulin.

Progesterone production during days 3–6 of culture tended ($P = 0.1$) to be greater by thecal cells from small follicles than by cells from large follicles, with intermediate production by cells from medium follicles (Fig. 5B). Both insulin and hCG stimulated progesterone produc-

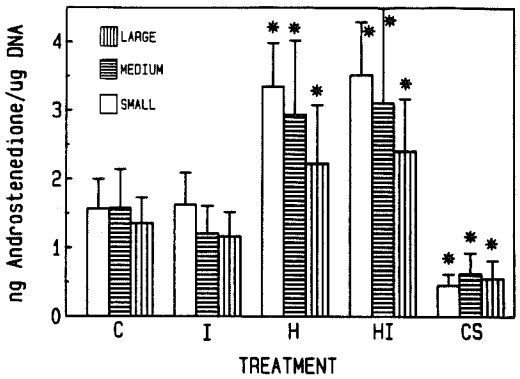

FIG. 4. Production of androstenedione during days 0–3 of culture of bovine thecal cells isolated from small (<5 mm; □), medium (5–10 mm; ▤), and large (>10 mm; ▥) follicles. Cells were cultured in the absence [control (C)] or presence of hCG (H), insulin (I), hCG and insulin (HI), and 10% (CS). Data are presented as nanograms of androstenedione per μg thecal cell DNA. Values with an *asterisk* are different ($P < 0.05$) from respective controls. Each *bar* represents the mean ± SEM from four experiments.

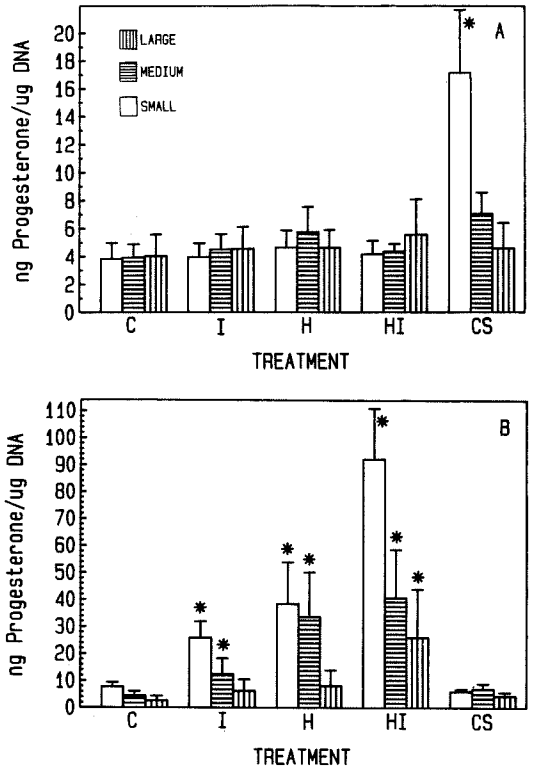

FIG. 5. Production of progesterone during days 0–3 of culture (A) and during days 3–6 of culture (B) of bovine thecal cells isolated from small (<5 mm; □), medium (5–10 mm; ▤), and large (>10 mm; ▥) follicles. Cells were cultured in the absence [control (C)] or presence of hCG (H), insulin (I), hCG and insulin (HI), and 10% calf serum (CS). Data are presented as nanograms of progesterone per μg thecal cell DNA. Values with an *asterisk* are different ($P < 0.05$) from respective controls. Each *bar* represents the mean ± SEM from four experiments.

tion in cells isolated from small and medium follicles, and the combination of the two hormones appeared to be additive during days 3–6 of culture. Treatment with hCG was more effective in stimulating progesterone production, with approximately a 6- to 8-fold increase *vs.* a 2- to 3-fold increase with insulin. Neither insulin nor hCG significantly stimulated progesterone production by cells from large follicles, but the combination of hCG and insulin did stimulate progesterone production (Fig. 5B). Combined results indicate that the capacity for progesterone production is greatest with cells from small follicles, intermediate with cells from medium follicles, and least with cells from large follicles. Cells from all stages of development were hormone responsive for androgen production; however, primarily cells from small and medium follicles produced progesterone in response to hormone stimulation.

To further characterize the hormonal responsiveness of thecal cells, dose-response curves for hCG and insulin were performed on a mixture of thecal cells from the different sized follicles. A minimum effective hCG concentration of 10 ng/ml stimulated androstenedione production during the first 3 days of culture, while the maximally effective concentration was between 0.1–1 μg/ml hCG (Fig. 6, *top panel*). Progesterone production between days 3–6 of culture was stimulated by a minimally effective concentration between 1–10 ng/ml, while the maximally effective concentration was between 10–100 ng/ml hCG (Fig. 6, *bottom panel*). Treatment of thecal cells with concentrations of insulin ranging from 5 ng to 5 μg/ml did not affect accumulation of androstenedione during days 0–3 of culture (data not shown). The effects of insulin on progesterone accumulation during days 3–6 of culture had a minimum effective concentration of 10 ng/ml insulin and maximum effective concentration of 100 ng/ml insulin (Fig. 7). Insulin-like growth factor-I (IGF-I) stimulated progesterone production by thecal cell cultures on day 6 of culture with concentrations between 1–10 ng/ml (data not shown). Combined results imply that hCG and insulin are active at nearly physiological concentrations.

The radiolabeled proteins secreted by the cultured thecal cells were examined to investigate more general cellular functions. Thecal cells were isolated from the different size follicles and cultured in the absence or

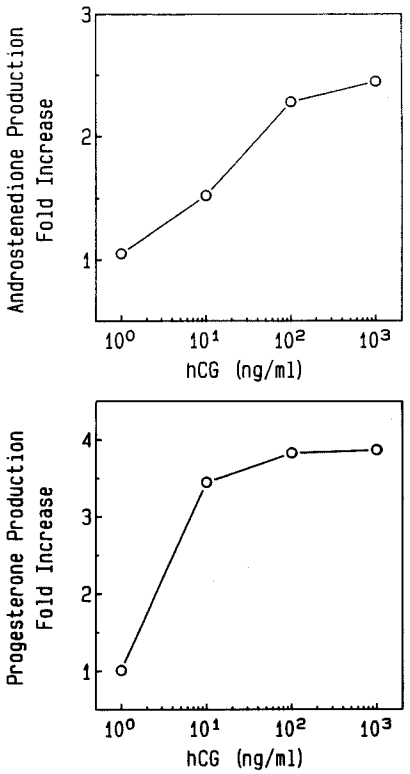

FIG. 6. Production of androstenedione during days 0–3 of culture (A) and of progesterone during days 3–6 of culture (B) in cultures of thecal cells treated with different doses of hCG (nanograms per ml). Data are presented as the fold increase over control nontreated cell cultures (nanograms per μg DNA). Each *point* represents the mean fold increase over control from three experiments.

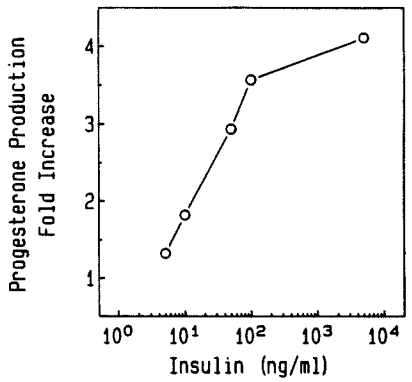

FIG. 7. Production of progesterone during days 3–6 of culture in cultures of thecal cells treated with different doses of insulin (nanograms per ml). Data are presented as the fold increase over control nontreated cell cultures (nanograms of progesterone per μg DNA). Each *point* represents the mean fold increase over control for three or more experiments.

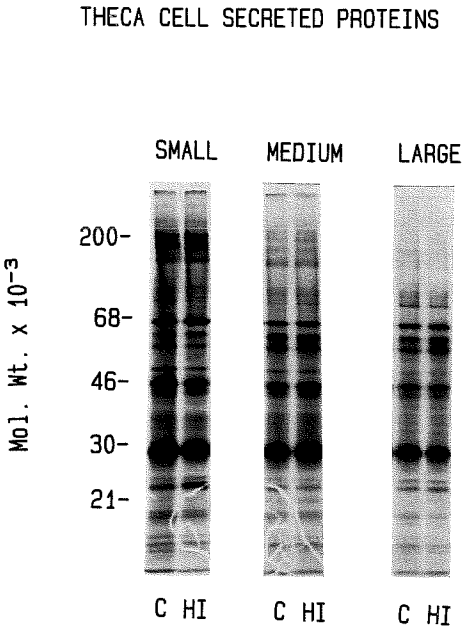

FIG. 8. Fluorograph of radiolabeled proteins secreted by thecal cells isolated from small (<5 mm), medium (5–10 mm), and large (>10 mm) follicles. Cells were cultured in glycine- and methionine-free medium containing 5 μCi [^{35}S]methionine/ml and 5 μCi [^{3}H]glycine/ml for 48 h. Radiolabeled secreted proteins were electrophoretically separated on SDS-polyacrylamide gradient (5–15%) slab gels and fluorographed. Cells were cultured in the absence [control (C)] or presence of hCG and insulin (HI). Data are representative of three experiments.

presence of hormones in medium containing radiolabeled methionine and glycine. Radiolabeled secreted proteins were collected from an equivalent number of cells, and profiles were examined on a fluorographed SDS-gel. The presence of hCG and insulin had little effect on the profiles of secreted proteins (Fig. 8). Major proteins between 20–70 kDa were consistently secreted from cells of all stages of development. Interestingly, proteins greater than 90 kDa were abundantly secreted from cells of small follicles, but their secretion was reduced from cells of medium follicles and further reduced or absent from cells of large follicles. Secretion of several proteins of 25, 20, and 15 kDa were also decreased with cells from large follicles (Fig. 8). Results indicate that while hormones have negligible effects on the secretion of proteins by thecal cells, the secretion of a number of proteins may be reduced as the follicle develops.

Discussion

The current study demonstrates that enzymatically dispersed bovine theca interna cells can be cultured in the absence of serum. Cells are viable for a minimum of 8 days of culture, with a decline in cell number observed during the first 3 days of culture. Analysis of the purity of the cell preparation demonstrates that more than 98% of the cell population has a stromal cell morphology, and only a 4% contamination with granulosa cells and/or endothelial cells was detected. The cytochemical procedure used to assess high levels of LDL receptor is anticipated to be a useful procedure to monitor the purity of thecal cell preparations. The lack of detectable aromatase activity in the thecal cell cultures confirms the absence of appreciable granulosa cell contamination. This serum-free culture of highly purified thecal cells was subsequently used to investigate the hormonal regulation of thecal cell function and differentiation.

Thecal cells were isolated from small, medium, and large antral follicles. Although only healthy developing follicles were selected, distinction between the follicle destined for ovulation and early atretic follicles is difficult. As previously discussed, follicles do not appear to be predestined for either ovulation or atresia, and follicles that would normally undergo atresia can be manipulated to ovulate (1–8). Therefore, a limitation of the current study that needs to be considered in data interpretation is that although healthy developing antral follicles were selected, not all follicles used would have ovulated. Preliminary experiments used a mixture of thecal cells from the different sized follicles to optimize culture conditions and establish appropriate time points to investigate the regulation of thecal cell function.

Comparison of thecal cells cultured in the presence or absence of serum indicates that although serum enhanced the ability to maintain cells in culture, serum dramatically altered cellular function and hormone responsiveness. Serum decreased androgen production and, with cells from small and medium follicles, increased progesterone production in the first few days of culture. The presence of serum also reduced the ability of hormones to stimulate progesterone production. Recently, Ikeda (48) reported that serum decreased androstenedi-

one production by mixed cultures of bovine theca interna and theca externa and that the ability of these cultures to produce progesterone in response to 8-bromo-cAMP was diminished in the presence of serum. However, Ikeda (48) also reported that the presence of serum in culture medium decreased the quantity of progesterone accumulation in untreated cultures, which is opposite the results obtained in the present studies. This discrepancy may be due to the fact that a mixture of theca interna and theca externa was cultured by Ikeda, whereas cultures of purified theca interna were used in the present study. In addition, the stimulatory effects of serum in the present study were only observed in theca interna from small or medium follicles during the first 3 days of culture, and Ikeda (48) used thecal cells from large follicles cultured for 6 days. Interestingly, secretion of androgen by thecal tissue from humans cultured in the presence of serum also declined during culture, and secretion of progesterone increased in response to serum (28). In contrast, accumulation of androstenedione in cultures of thecal explants from rats in the presence of 10% fetal calf serum increased from days 2–6 of culture, and the increase was even greater in the presence of LH (20). This response in the rat may be due in part to substances present in serum, since culture of rat thecal explants in 1% serum required LH to maintain androstenedione production (22). Combined results from these studies and the present observations demonstrate that the effect of serum on thecal cell function needs to be considered when serum-supplemented cultures are used to investigate hormonal regulation of thecal cell function. For these reasons, subsequent experiments involved serum-free cultures of thecal cells.

Additional observations made during the present studies indicate that thecal cell production of androgens and progesterone changed during culture. These alterations in cellular function may reflect a change in cellular differentiation that appears to be similar to the functional changes that occur *in vivo* as the follicle undergoes luteinization, and progesterone production predominates over androgen production. Basal and hormonally stimulated production of androgens was greatest during the first 3 days of culture, and accumulation of androstenedione was approximately 10-fold higher than testosterone. The predominance of thecal cell production of androstenedione over testosterone has previously been reported in bovine (49), humans (28), swine (24, 35), and rats (32, 50). After 3 days of culture, androgen production declined dramatically and was not maintained by hCG treatment. Conversely, basal progesterone production remained relatively constant through day 6 of culture, and hormones had little effect on progesterone production until after 3 days of culture. This change in the steroidogenic capacity of the thecal cell observed between days 1–3 and days 4–6 of culture may represent a switch in predominance of the Δ^5 steroidogenic pathway, the major pathway for androgen production by bovine thecal cells (13, 51), to the Δ^4 steroidogenic pathway due to changes in expression and/or activity of cytochrome P450 side-chain cleavage enzyme (50), 3β-hydroxysteroid dehydrogenase (13), and/or cytochrome $P450_{17}\alpha$ enzyme, an enzyme with both 17α-hydroxylase and 17–20-lyase activity (10, 11). Results imply that bovine thecal cells appear to develop functional characteristics of a luteinization-like stage of development during culture. Similar observations have been made with cultures of rat thecal/interstitial cells (22), bovine theca interna and externa (48), and granulosa cells from the bovine (41) and rat. The 17α-hydroxylase/lyase enzymatic system has previously been shown to be sensitive to long term cell culture and decline during luteinization (9). This is also reflected in the decline in hormone responsiveness of cells from large follicles. Therefore, conclusions regarding potential luteinization of thecal cells in culture *vs.* an altered steroidogenic capacity due to unstable steroidogenic enzymes will require further investigation.

Analysis of the hormonal regulation of thecal cell steroidogenesis revealed that hCG has an important role in regulating both androgen and progesterone production by thecal cells. Treatment with hCG stimulated androgen production from days 0–3 of culture and progesterone production from days 3–6 of culture. Since hCG mimics the effects of LH (33), these findings confirm previous observations made in a number of species (20–22, 30, 33, 38) which demonstrate thecal cell steroidogenesis is maintained and controlled by LH or hCG. In contrast to the effects of hCG on thecal cell steroidogenesis, hCG had no detectable effects on a more general analysis of thecal cell function, the secretion of radiolabeled proteins. A large number of minor and major thecal cell secreted proteins were observed between 5–500 kDa. Treatment with hCG alone or in combination with insulin had no apparent effect on secretion of radiolabeled proteins by thecal cell isolated from various stages of development. Therefore, the actions of hCG/LH on thecal cell differentiation may be selective to specific functions rather than a stimulation of general cellular function. Although hCG may play a critical role in the regulation of thecal cell steroidogenesis, effects on other cellular functions remain to be investigated.

The actions of insulin on thecal cell function were also investigated and found to be different for androgen and progesterone production. Insulin had no effect on androgen production throughout the culture period. However, insulin increased progesterone production during days 3–6 of culture, and the combination of insulin and hCG appeared additive with respect to progesterone production. Previous studies have also demonstrated that in-

sulin does not alter androstenedione production in cultures of bovine theca interna and externa cells (48) and that insulin increases progesterone production by thecal cells from bovine (48) and porcine (52, 53). However, these results differ from those in other species where insulin was reported to stimulate androstenedione production by porcine theca (52, 53) and rat thecal-interstitial cells (31). Insulin has also been shown to enhance hCG actions (31, 52, 53) and to prolong the stimulatory effects of hCG on androstenedione production by rat thecal-interstitial cells (54). The reason for these apparent species differences regarding insulin's effects on androgen production remains to be determined.

The dose of insulin used in the time-course and developmental experiments was supraphysiological (5 μg/ml). Therefore, the responses of insulin in these studies could possibly be mediated through the IGF-I receptor. Data obtained from the dose-response studies indicate that a dose of 10 ng insulin/ml effectively stimulated progesterone. Studies on rat thecal-interstitial cells (31) and porcine theca (52) demonstrate that insulin can stimulate steroidogenesis at physiological concentrations and that rat thecal interstitial cells contain insulin receptors (31). However, physiological concentrations of IGF-I were more effective at stimulating androgen production in these and other studies (51). Physiological concentrations of IGF-I were also effective in stimulating progesterone production in the present study (data not shown). Therefore, results of the current study indicate that insulin may directly regulate thecal cell steroidogenesis *in vivo*; however, the physiological relevance of the actions of insulin *vs.* IGF-I remains to be determined.

Analysis of the developmental regulation of thecal cell function revealed that basal and hCG-stimulated androstenedione accumulation were relatively constant for the different follicle sizes. Secretion of androstenedione by thecal explants from bovine (21, 49) and humans (28) have also been shown to be relatively constant per unit mass of tissue from different sized follicles. However, results in the pig (24, 35) and rat (20, 22) indicate that androstenedione production increases with increased follicular development. The differences between these studies may be due to species differences or the fact that the experimental protocols used for obtaining different stages of development in the swine and rat involved gonadotropin treatment of animals, a procedure reported to result in different hormonal profiles than those observed in normal cycling sows (55). Follicular development in these studies were dependent on duration of gonadotropin treatment before collection. The bovine thecal cell culture system developed in the present study eliminates this variable.

Progesterone production did not differ by thecal cells isolated from different sized follicles during the first 3 days of culture. However, during days 3–6 of culture basal secretion of progesterone tended to be reduced in thecal cells from large follicles. Secretion of progesterone in response to hCG and/or insulin also decreased with increasing follicle size, especially in theca from large follicles. These findings are similar to those previously observed in swine (35), but differ from those in the rat (22). Results imply that the capacity of thecal cells from different sized follicles to produce progesterone may change over time during *in vitro* culture.

To examine the developmental regulation of more general functions of thecal cells, the secretion of radiolabeled proteins was evaluated. Secretion of high mol wt proteins as well as several low mol wt proteins diminished with increased follicular development. Although the high mol wt proteins were not characterized in this study, the sizes of these proteins are similar to those of proteins involved in formation of the extracellular matrix. The importance of continued secretion of these proteins may decline as the follicle increases in size. As discussed previously, hCG and insulin had little effect on the profiles of secreted proteins. These findings are different from those in bovine granulosa cells, where no major differences in protein secretion were apparent between different stages of follicle development, but FSH and insulin stimulated the synthesis of several proteins by granulosa cells isolated from small, medium, and large follicles (41). Combined results indicate that regulation of protein secretion by thecal cells during follicle development may be independent of hCG and/or insulin regulation, but functional changes in granulosa cell protein secretion during follicle development are regulated by FSH and/or insulin.

Collectively, the data obtained in this and other studies provide evidence that gonadotropin and/or insulin regulation of thecal cell differentiation during development of small antral follicles to large follicles may not be as crucial for ovarian physiology as it is to regulate granulosa cell differentiation. This hypothesis is supported by the absence of major differences in hCG and/or insulin stimulation of androgen production and protein secretion between the different stages of follicular development. In contrast, FSH and/or insulin stimulation of aromatase activity and progesterone production by granulosa cells was enhanced with increased follicular development (41). Furthermore, the capacity of theca from small antral follicles to respond to LH and synthesize androstenedione precedes the ability of granulosa to metabolize androgen to estrogen (21). The observations imply that gonadotropin and/or insulin stimulation may be more important in regulating differentiation of bovine thecal cells during the early stages of development of preantral and small antral follicle as well as during luteinization.

In addition to the role of gonadotropin and/or insulin

in regulating thecal cell function and differentiation, many other paracrine factors may be involved in regulating or promoting functional changes in thecal cells during follicular development. Coculture of bovine granulosa and thecal cells from preovulatory follicles results in an increase in androstenedione production over that by thecal cells cultured in the absence of granulosa cells (13). This paracrine effect has been shown to be mediated by both estradiol and pregnenolone (13, 23). Observations presented elsewhere demonstrate that estradiol can cause a more dramatic increase in thecal secretion of androstenedione than hCG (56), indicating that estrogen may be equally important as hCG in regulating thecal cell differentiation. Further studies are needed to determine the role of ovarian steroids in the regulation of thecal cell function and differentiation.

In summary, results from the present study demonstrate that bovine thecal cells can be cultured under serum-free conditions and that the presence of serum may interfere with investigations of the hormonal and developmental regulation of bovine thecal cell function. Observations demonstrate an apparent differentiation of thecal cells during culture, which may parallel changes that take place during luteinization *in vivo*. The changes during culture are associated with differential effects of insulin and hCG on steroidogenesis. Although no major developmental responses to hCG and insulin were observed, it is apparent that developmental changes in thecal cell function do exist, providing support for the use of the bovine as an animal model for studies concerning thecal cell differentiation during follicular development. The similarities of thecal cell function observed in the present studies with previous findings in the human indicate that the bovine may be a useful model for obtaining information that could be applied to human follicular development.

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References

1. Spicer LJ, Echternkamp SE 1986 Ovarian follicular growth, function and turnover in cattle: a review. J Anim Sci 62:428
2. Savio JD, Keenan L, Boland MP, Roche JF 1988 Pattern of growth of dominant follicles during the oestrous cycle in heifers. J Reprod Fertil 83:663
3. Sirois J, Fortune JE 1988 Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 39:308
4. Ginter OJ, Knopf L, Kastelic JP 1989 Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil 87:223
5. Rahmanian MS, Murdoch WJ 1987 Function of ovine CL after administration of LHRH. J Anim Sci 64:648
6. Baird DT, Swanston IA, McNeilly AS 1981 Relationship between LH, FSH and prolactin concentrations and the secretion of androgens and estrogens by the preovulatory follicle in the ewe. Biol Reprod 24:1013
7. Draincourt MA, Bodin L, Boomarov O, Thimonier J, Elsen JM 1990 Number of mature follicles ovulation after a challenge of human chorionic gonadotropin in different breeds of sheep at different physiological stages. J Anim Sci 68:719
8. Tsonis CG, Cahil LP, Carson RS, Findlay JK 1984 Identification at the onset of luteolysis of follicle capable of ovulation in the ewe. J Reprod Fertil 70:609
9. Richard JS, Jahnsen T, Hedin L, Lifka J, Ratoosh S, Durica JM, Goldring NB 1987 Ovarian follicular development: from physiology to molecular biology. Recent Prog Horm Res 43:231
10. Hedin L, Rodgers RJ, Simpson ER, Richards JS 1987 Changes in content of cytochrome $P450_{17\alpha}$, cytochrome P450scc, and 3-hydroxy-3-methylglutaryl CoA reductase in developing rat ovarian follicles and corpora lutea: correlation with thecal cell steroidogenesis. Biol Reprod 37:211
11. McAllister JM, Kerin JFP, Trant JM, Estabrook RW, Mason JI, Waterman MR, Simpson ER 1989 Regulation of cholesterol side-chain cleavage and 17α-hydroxylase/lyase activities in proliferating human theca interna cells in long term monolayer culture. Endocrinology 125:1959
12. Dorrington JH, Moon YS, Armstrong DT 1975 Estradiol-17β biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone. Endocrinology 97:1328
13. Fortune JE 1986 Bovine theca and granulosa cells interact to promote androgen production. Biol Reprod 35:292
14. Skinner MK, Keski-Oja J, Osteen K, Moses HL 1987 Ovarian theca cells produce transforming growth factor-β which can regulate granulosa cell growth. Endocrinology 121:786
15. Skinner MK, Coffey RJ 1988 Regulation of ovarian cell growth through the local production of transforming growth factor-α by theca cells. Endocrinology 123:2632
16. Adashi EY, Resnick CE, Twardzik DR 1987 Transforming growth factor-α attenuates the acquisition of aromatase activity by cultured rat granulosa cells. J Cell Biochem 33:1
17. Knecht M, Feng P, Catt K 1987 Bifunctional role of transforming growth factor-β during granulosa cell development. Endocrinology 120:1243
18. Dodson WC, Schomberg DW 1987 The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120:512
19. Mondschein JS, Canning SF, Hammond JM 1988 Effect of transforming growth factor-β on the production of immunoreactive insulin-like growth factor I and progesterone on [^{3}H]thymidine incorporation in porcine granulosa cell cultures. Endocrinology 123:1970
20. Bogovich K, Scales LM, Higginbottom E, Ewing LL, Richards JS 1986 Short term androgen production by rat ovarian follicles and long term theca steroidogenesis by explants in culture. Endocrinology 118:1379
21. McNatty KP, Heath DA, Henderson KM, Lun S, Hurst PR, Ellis LM, Montgomery GW, Morrison L, Thurley DC 1984 Some aspects of thecal and granulosa cell function during follicular development in the bovine ovary. J Reprod Fertil 72:39
22. Richards JS, Hedin L, Caston L 1986 Differentiation of rat ovarian thecal cells: evidence for functional luteinization. Endocrinology 118:1660
23. Fortune JE, Hansel W 1979 The effect of 17β-estradiol on progesterone secretion by bovine theca and granulosa cells. Endocrinology 104:1834
24. Evans G, Dobias M, King GJ, Armstrong DT 1981 Estrogen, androgen, and progesterone biosynthesis by theca and granulosa of preovulatory follicles in the pig. Biol Reprod 25:673
25. Haney AF, Schomberg DW 1981 Estrogen and progesterone production by developing porcine follicles *in vitro*: evidence for estrogen formation by theca. Endocrinology 109:971

26. Channing CP 1980 Progesterone and estrogen secretion by cultured monkey ovarian cell types: influences of follicular size, serum luteinizing hormone levels, and follicular fluid estrogen levels. Endocrinology 107:342
27. Batta SK, Wentz AC, Channing CP 1980 Steroidogenesis by human ovarian cell types in culture: influence of mixing cell types and effects of added testosterone. J Clin Endocrinol Metab 50:274
28. McNatty KP, Makris A, DeGrazia C, Osathanondh R, Ryan KJ 1979 The production of progesterone, androgens and estrogens by granulosa cells, theca tissue and stromal tissue from human ovaries *in vitro*. J Clin Endocrinol Metab 49:687
29. Moon YS, Tsang BK, Simpson C, Armstrong DT 1978 17β-Estradiol biosynthesis in cultured granulosa and thecal cells of human ovarian follicles: stimulation by follicle-stimulating hormone. J Clin Endocrinol Metab 47:263
30. Dennefors BL, Nilsson L, Hamberger L 1982 Steroid and adenosine 3′,5′-monophosphate formation in granulosa and thecal cells from human preovulatory follicles in response to human chorionic gonadotropin. J Clin Endocrinol Metab 54:436
31. Hernandez ER, Resnick CE, Holtzclaw WD, Payne DW, Adashi EY 1988 Insulin as a regulator of androgen biosynthesis by cultured rat ovarian cells: cellular mechanism(s) underlying physiological and pharmacological hormone actions. Endocrinology 122:2034
32. Magoffin DA 1989 Transforming growth factor-β promotes differentiation of ovarian thecal-interstitial cells but inhibits androgen production. Endocrinology 125:1951
33. Magoffin DA, Erickson GF 1982 Primary culture of differentiating ovarian androgen-producing cells in defined medium. J Biol Chem 257:4507
34. Skinner MK, Lobb D, Dorrington JH 1987 Ovarian thecal/interstitial cells produce an epidermal growth factor-like substance. Endocrinology 121:1892
35. Tsang BK, Ainsworth L, Downey BR, Marcus GJ 1985 Differential production of steroids by dispersed granulosa and theca interna cells from developing preovulatory follicles of pigs. J Reprod Fertil 74:459
36. Stoklosowa S, Gregoraszczuk E, Channing CP 1982 Estrogen and progesterone secretion by isolated cultured porcine thecal and granulosa cells. Biol Reprod 26:943
37. Caubo B, DeVinna RS, Tonetta SA 1989 Regulation of steroidogenesis in cultured porcine theca cells by growth factors. Endocrinology 125:321
38. Tonetta SA, DeVinna RS, diZeregra GS 1986 Modulation of porcine thecal aromatase activity by human chorionic gonadotropin, progesterone, estradiol-17β, and dihydrotestosterone. Biol Reprod 35:785
39. Porter TE, Hargis BM, Silsby JL, Halawani MEE 1989 Differential steroid production between theca interna and theca externa cells: a three-cell model for follicular steroidogenesis in avian species. Endocrinology 125:109
40. Tilly JL, Johnson AL 1989 Regulation of androstenedione production by adenosine 3′,5′-monophosphate and phorbol myristate acetate in ovarian thecal cells of the domestic hen. Endocrinology 125:1691
41. Skinner MK, Osteen KG 1988 Developmental and hormonal regulation of bovine granulosa cell function in the preovulatory follicle. Endocrinology 123:1668
42. Metcalf MG 1982 Estimation of viability of bovine granulosa cells. J Reprod Fertil 65:425
43. Goldstein JL, Ho YK, Basu SK, Brown MS 1979 Binding site on macrophages that mediate uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 76:333
44. Skinner MK, Fetterolf P, Anthony CT 1988 Purification of a paracrine factor, PModS, produced by testicular peritubular cells that modulate Sertoli cell function. J Biol Chem 263:2884
45. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:280
46. Skinner MK, Griswold MD 1983 Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem J 209:281
47. Osteen KG, Anderson LD, Reichert Jr LE, Channing CP 1985 Follicular fluid modulation of functional LH receptor induction in pig granulosa cells. J Reprod Fertil 74:407
48. Ikeda H 1990 Serum-free medium conditions for steroidogenesis of bovine follicular thecal cells cultured on collagen gel matrix. In Vitro Cell Dev Biol 26:193
49. McNatty KP, Heath DA, Lun S, Fannin J, McDairmid JM, Henderson KM 1984 Steroidogenesis by bovine theca interna in an *in vitro* perfusion system. Biol Reprod 30:159
50. Magoffin DA, Gancedo B, Erickson GF 1989 Transforming growth factor-β promotes differentiation of ovarian thecal/interstitial cells but inhibits androgen production. Endocrinology 125:1951
51. Lacroix E, Eechaute W, Leusen I 1974 The biosynthesis of estrogens by cow follicles. Steroids 23:337
52. Morley P, Calaresu FR, Barbe GJ, Armstrong DT 1989 Insulin enhances luteinizing hormone-stimulated steroidogenesis by porcine theca cells. Biol Reprod 40:735
53. Barbieri RL, Makris A, Ryan KJ 1983 Effects of insulin on steroidogenesis in cultured porcine ovarian theca. Fertil Steril 40:237
54. Magoffin DA, Erickson GF 1988 An improved method for primary culture of ovarian androgen-producing cells in serum-free medium: effect of lipoproteins, insulin, and insulin-like growth factor-I. In Vitro Cell Dev Biol 24:862
55. Babaloa GO, Shapiro BH 1988 Correlation of follicular steroid hormone profiles with ovarian cyclicity in sows. J Reprod Fertil 84:79
56. Roberts AJ, Skinner MK 1990 Estrogen regulation of theca cell steroidogenesis and differentiation: theca cell-granulosa cell interactions. Endocrinology 127:2918