

# Effects of Insulin, Insulin-Like Growth Factor I, and Gonadotropins on Bovine Granulosa Cell Proliferation, Progesterone Production, Estradiol Production, and(or) Insulin-Like Growth Factor I Production In Vitro<sup>1</sup>

L. J. Spicer<sup>\*,2</sup>, E. Alpizar<sup>\*</sup>, and S. E. Echtenkamp<sup>†</sup>

<sup>\*</sup>Department of Animal Science, Oklahoma State University, Stillwater 74078 and

<sup>†</sup>Roman L. Hruska U.S. Meat Animal Research Center, ARS, USDA, Clay Center, NE 68933-0166

**ABSTRACT:** The objectives of the present studies were to determine the effect of insulin, insulin-like growth factor I (IGF-I), testosterone, and FSH on proliferation, progesterone production, and(or) estradiol production of bovine granulosa cells. In addition, existence of IGF-I mRNA in granulosa cells and in vitro IGF-I production by granulosa cells were assessed. Cells from small (1 to 5 mm) and large ( $\geq 8$  mm) follicles were collected from cattle and cultured for either 3 or 4 d. When cells from small follicles were cultured, insulin (.1 to 10  $\mu\text{g}/\text{mL}$ ) and IGF-I (100 to 400 ng/mL) increased ( $P < .05$ ) cell numbers compared with controls. Insulin alone or IGF-I alone increased ( $P < .05$ ) progesterone production per cell

by severalfold on d 4. In cells from both sizes of follicles, insulin (1  $\mu\text{g}/\text{mL}$ ), in the presence of FSH, increased estradiol production per cell. In contrast, IGF-I (100 ng/mL) inhibited estradiol production by cells from small follicles and stimulated estradiol production by cells from large follicles. Insulin-like growth factor II (100 ng/mL) and insulin at higher doses ( $\geq 5 \mu\text{g}/\text{mL}$ ) had no effect on estradiol production by cells from small and large follicles. Granulosa cells contained four IGF-I mRNA transcripts and produced IGF-I in vitro. These results support the hypothesis that insulin and IGF-I may have direct local effects on bovine ovarian function, and that these effects are influenced by dose and size of follicle.

Key Words: IGF-I, Gonadotropins, Granulosa Cells, Graafian Follicles, Progesterone, Cattle

J. Anim. Sci. 1993. 71:1232-1241

## Introduction

In addition to their known metabolic effects, insulin and insulin-like growth factor I (IGF-I) have been shown to stimulate both progesterone production and

mitosis of bovine ovarian granulosa cells cultured in vitro (Savion et al., 1981; Schams et al., 1988; Langhout et al., 1991). Evidence for rats, pigs, and humans indicates that IGF-I can be produced by granulosa cells and thus may act as a local regulator of ovarian function (Adashi et al., 1989; Geisthovel et al., 1990; Hammond et al., 1991). In vivo studies indicate that concentrations of IGF-I increase with increased follicular size in cattle (Spicer et al., 1988; Spicer and Enright, 1991) and pigs (Bryan et al., 1989). In vivo data also reveal a positive correlation between follicular fluid IGF-I and progesterone concentrations in postpartum anestrous and cyclic cows (Spicer et al., 1988; Echtenkamp et al., 1990; Spicer and Enright, 1991). A positive correlation between follicular fluid IGF-I and estradiol was found in two of three studies (Spicer et al., 1988; Echtenkamp et al., 1990; Spicer and Enright, 1991). However, reports on direct effects of insulin and IGF-I on aromatase activity of bovine ovarian cells are meager. In addition, production of IGF-I by bovine granulosa cells

<sup>1</sup>This research was supported in part by the Oklahoma Agric. Exp. Sta. (Journal Article no. 6144). The authors thank the National Hormone and Pituitary Program (Univ. of Maryland School of Medicine, Baltimore, MD) for supplying IGF-I antiserum, oFSH, bLH, and bGH; Wellington Quality Meats (Wellington, KS) and Ralph's Packing Co. (Perkins, OK) for their generous donations of bovine ovaries; P. Rotwein (Washington Univ. School of Medicine, St. Louis, MO) for providing the IGF-I cDNA probe; and R. Smith and T. Boman for expert technical assistance. Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

<sup>2</sup>To whom correspondence should be addressed.

Received September 11, 1992.

Accepted December 15, 1992.

has not been reported. Therefore, we set out to determine the effects of insulin, IGF-I, and other trophic factors on granulosa cell growth, progesterone production, and aromatase activity under serum-free culture conditions, as well as to measure granulosa cell IGF-I production in vitro and IGF-I mRNA levels in bovine granulosa cells.

## Materials and Methods

**Reagents and Hormones.** Reagents were Dulbecco's Modified Eagles Medium (DMEM), Ham's F10, fetal calf serum (FCS), bovine insulin, and estradiol (E<sub>2</sub>) from Sigma Chemical (St. Louis, MO); bovine LH (USDA-bLH-B5, LH activity  $2.1 \times$  NIH-LH-S1 U/mg; FSH activity  $< 1.0\%$  by weight), bovine growth hormone (GH) (USDA-bGH-B1, GH activity  $1.9 \times$  NIH-GH-B18 U/mg; prolactin activity  $< 10\%$  by weight; FSH activity  $< .06\%$  by weight) and ovine FSH (NIADDK-oFSH-17, FSH activity  $20 \times$  NIH-FSH-S1 U/mg; LH activity  $.04 \times$  NIH-LH-S1 U/mg; GH activity  $< .1\%$  by weight) from National Hormone and Pituitary Program (Baltimore, MD); epidermal growth factor (EGF), recombinant human IGF-I, and recombinant human IGF-II from Amgen Biologicals (Thousand Oaks, CA); [<sup>125</sup>I]iodo-estradiol and [<sup>125</sup>I]iodo-progesterone from ICN Biomedicals (Costa Mesa, CA); and testosterone from Steraloids (Wilton, NH).

**Cell Culture.** Ovaries were obtained at nearby commercial abattoirs from beef and dairy cows and heifers after slaughter. After transport to the laboratory on ice ( $< 120$  min), the ovaries were processed as previously described (Langhout et al., 1991). Briefly, ovaries were washed three times in saline (.15 M NaCl), immersed in 70% ethanol for 30 s, and then washed again three times with saline. The ovaries were kept on ice in saline until the granulosa cells were collected. Follicles were separated into two groups based on surface diameter: small (1 to 5 mm) and large ( $\geq 8$  mm). Granulosa cells were collected by aspiration using a needle (20 gauge, 38.1 mm) and syringe (plastic, 3 mL) and washed three times in serum-free medium. At each wash, cells were separated from medium via centrifugation ( $200 \times g$  for 10 min) and maintained at 4°C. Medium was a 1:1 mixture of DMEM and Ham's F10 containing .12 mM gentamicin and 38.5 mM sodium bicarbonate. Numbers of viable cells ( $27 \pm 3\%$  and  $38 \pm 4\%$  of total cells obtained from small and large follicles, respectively) were determined using the trypan blue exclusion method. Between 2 and  $4 \times 10^5$  viable cells in 35 to 90  $\mu$ L of medium were added to Falcon multiwell plates (#3047, Becton Dickinson, Oxnard, CA) containing 1 mL of medium. Cultures were kept at 37°C in a 5% CO<sub>2</sub> atmosphere, and medium was changed every day as previously described (Langhout et al., 1991). To

obtain optimal attachment, cells were maintained in the presence of 10% FCS for the first 2 d of culture, unless stated otherwise. After this time, granulosa cells were washed twice with .5 mL of serum-free medium and incubations continued in serum-free medium with or without added hormones. For studies evaluating the effects of hormones on cell proliferation and progesterone production, hormonal treatments were applied for an additional 2 d (i.e., from d 2 to 4 of culture), and cells from only small follicles were used because of the limited number of large follicles (used for aromatase studies) on ovaries. For studies evaluating the effects of hormones on aromatase activity, hormonal treatments were applied for 1 d (i.e., from d 2 to 3 of culture), unless stated otherwise.

**Estradiol Production.** Estradiol production (i.e., functional aromatase activity) was assessed during a 24-h exposure of granulosa cells to 1 or 3  $\mu$ g/mL of testosterone used for an estrogen precursor as previously described (Spicer et al., 1992a). After the 24-h incubation, concentrations of estradiol in medium were determined by RIA and cell numbers were determined. Estradiol production was expressed as picograms of estradiol  $\cdot 10^5$  cells<sup>-1</sup>  $\cdot 24$  h<sup>-1</sup>.

**Determination of Numbers of Granulosa Cells.** Numbers of granulosa cells were determined at the termination of experiments using a Coulter counter (Model Zm, Coulter Electronics, Hialeah, FL) as previously described (Baranao and Hammond, 1985; Langhout et al., 1991). Cells were exposed to .5 mL of trypsin (.25% wt/vol) for 30 min at 25°C, and then scraped from each well, diluted in .15 M NaCl, and enumerated.

## Radioimmunoassays

**Progesterone.** Concentrations of progesterone in culture medium collected on d 4 of culture were determined by RIA as previously described (Baranao and Hammond, 1985). The intra- and interassay CV were 11.1 and 17.2%, respectively. The doses of testosterone and the other hormones used did not cross-react in the assay.

**Estradiol.** Concentrations of estradiol in culture medium were determined by RIA as previously described (Spicer and Enright, 1991). The intra- and interassay CV were 10.5 and 22.6%, respectively. The doses of testosterone and the other hormones used did not cross-react in the assay.

**Insulin-Like Growth Factor I.** Concentrations of IGF-I in culture medium were determined by RIA as previously described (Echternkamp et al., 1990). Briefly, aliquots of medium were diluted 1:4 with 87.5% acidic ethanol (.25 N HCl final concentration) and incubated for 16 h at 4°C. Samples were then centrifuged for 30 min at  $1,200 \times g$  at 4°C and neutralized with .855 M Tris. The extract was then concentrated three- to sixfold using Centricon-3 (molecular weight cut-off = 3,000) microconcentrators

(Amicon, Danvers, MA); aliquots from each concentrated sample were included in the assay. This procedure resulted in parallelism between the human IGF-I standard and the extracted, concentrated culture medium.

**RNA Isolation and mRNA Measurement.** Cows were treated with FSH (total dosage = 32 mg of FSH-P; administered twice daily starting at the midluteal phase of an estrous cycle) in decreasing dosages for 4 d. Granulosa cells from  $\geq 6$ -mm follicles and liver tissue were collected immediately at slaughter 24 h after prostaglandin  $F_{2\alpha}$  administered on the 4th d of the FSH treatment. Tissues were frozen in liquid nitrogen immediately after slaughter. Tissue was thawed and homogenized in Solution D containing 4 M guanidine isothiocyanate, .1 M 2- $\beta$ -mercaptoethanol, .5% N-lauryl-sarkosine, and 25 mM sodium citrate (Chomczynski and Sacchi, 1987). Total cellular RNA was obtained by double precipitation in 100% ethanol, washed in 80% ethanol, and vacuum-dried. A human IGF-I (hIGFA 818 BP) complementary DNA (cDNA) was used to probe granulosa cell and liver tissue RNA to determine IGF-I mRNA levels (Rotwein, 1986). Briefly, samples (40  $\mu$ g/lane) of total cellular RNA were loaded onto 1.2% agarose gels containing formaldehyde and ethidium bromide and subjected to electrophoresis. The gels were then examined under UV transillumination to assess the quality of the RNA preparations. The RNA was then transferred to nitrocellulose filters and hybridized with the IGF-I cDNA probe as described previously (Burke et al., 1991). After hybridization, membranes were washed in  $5 \times$  saline sodium citrate (SSC; containing .75 M NaCl, .075 M sodium citrate, and .1% SDS) for 15 min at 24°C and then in  $.2 \times$  SSC (containing .03 M NaCl, .003 M sodium citrate, and .1% SDS) for 15 min at 52°C, and autoradiographed for 7 d at -70°C. Duplicate blots were probed with a constitutive probe,  $\beta$ -actin, to confirm that the RNA quantity was similar among preparations.

**Statistical Analyses.** Experimental data are presented as the least squares means  $\pm$  SE of measurements from quadruplicate culture wells from two or more experiments. Each experiment was performed two or three times with different pools of granulosa cells collected from 12 to 64 ovaries for each pool. Treatment effects and interactions were assessed using the GLM procedure of SAS (1988). Main effects were treatment and experiment when data from more than one experiment were analyzed. Each well was a replicate and each experiment contained four replicates per treatment. Progesterone and estradiol production were expressed as nanograms or picograms  $\cdot 10^5$  cells $^{-1} \cdot 24$  h $^{-1}$ , with cell numbers at the termination of the experiment used for this calculation. Specific differences in cell growth, progesterone production, and estradiol production between treatments were determined using the Fisher's protected LSD procedure (Ott, 1977).

## Results

### *Effect of Various Hormones on Granulosa Cell Proliferation and Progesterone Production*

**Effect of Insulin and Insulin-Like Growth Factor I on Granulosa Cells from Small Follicles.** To determine the dose response of insulin and IGF-I on granulosa cell proliferation and progesterone production,

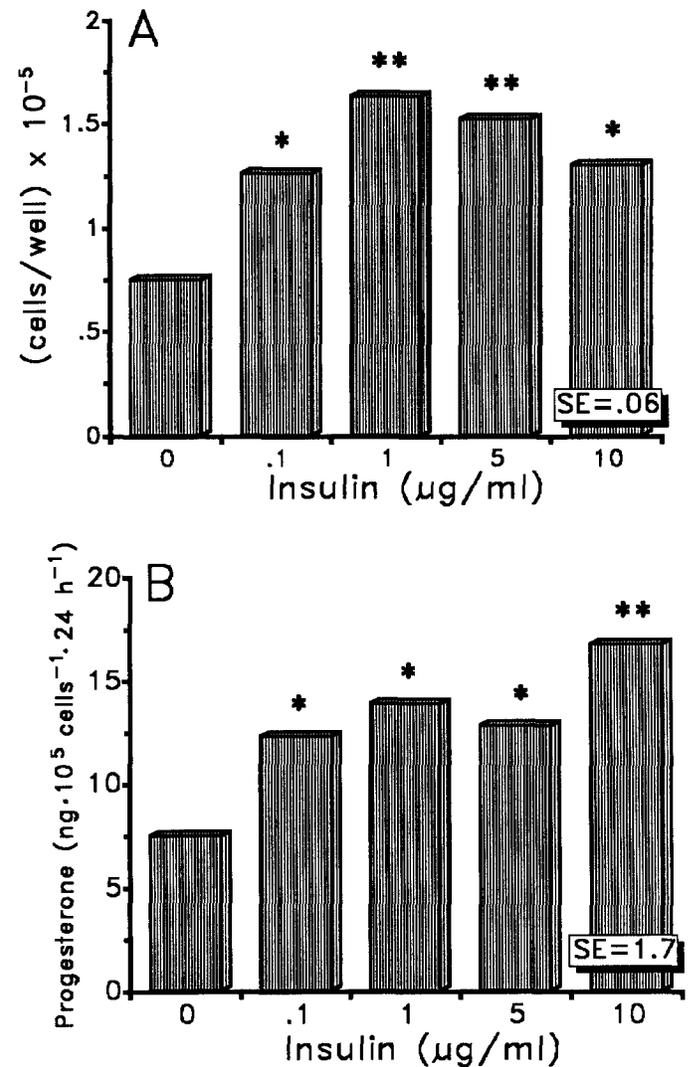


Figure 1. Dose-response of insulin on granulosa cell proliferation (Panel A) and progesterone production (Panel B). Cells from small bovine follicles were cultured for 2 d in the presence of 10% fetal calf serum and then treated with control or insulin (.1 to 10  $\mu$ g/mL) for an additional 2 d. Medium was changed every 24 h. Progesterone concentrations in medium and cell numbers were determined on d 4. Values are least squares means of quadruplicate cultures from three separate experiments. Pooled SE =  $.06 \times 10^5$  cells per well for Panel A and  $1.7 \text{ ng} \cdot 10^5 \text{ cells}^{-1} \cdot 24 \text{ h}^{-1}$  for Panel B. \*Mean differs from control ( $P < .05$ ). \*\*Mean differs from .1  $\mu$ g/mL of insulin ( $P < .05$ ).

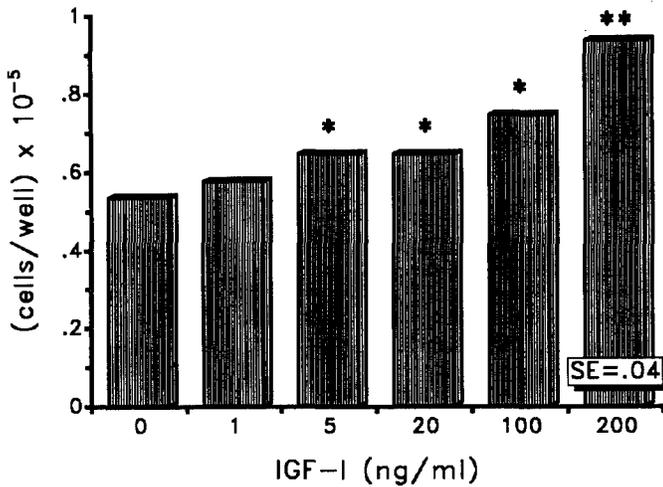


Figure 2. Dose-response of insulin-like growth factor I (IGF-I) on granulosa cell proliferation. Cells from small follicles were cultured as described in legend for Figure 1. Pooled SE = .04 × 10<sup>5</sup> cells per well. \*Mean differs from control (*P* < .05). \*\*Mean differs from 5 ng/mL of IGF-I (*P* < .05).

granulosa cells from small follicles were cultured for 2 d in 10% FCS followed by 2 d in serum-free medium containing control (no additions), insulin (.1 to 10 μg/mL), or IGF-I (1 to 400 ng/mL). On d 4 of culture, medium was collected for progesterone analysis and cell numbers were determined. Cell numbers were greater (*P* < .05) for insulin-treated cultures than for controls (Figure 1A). Insulin treatment (.1 to 10 μg/mL) also increased (*P* < .05) progesterone production compared with controls (Figure 1B). As low as 5 ng/mL of IGF-I increased (*P* < .05) cell numbers above controls (Figure 2); 200 ng/mL of IGF-I caused a further increase (*P* < .05) in cell numbers above that seen with 5 ng/mL of IGF-I. In two additional experiments in which 200 and 400 ng/mL of IGF-I were compared, 400 ng/mL of IGF-I did not increase (*P* > .10) cell numbers above those of 200 ng/mL of IGF-I (1.01 vs .89 ± .06 × 10<sup>5</sup> cells/well). Progesterone production was greater (*P* < .05) in cultures treated with FSH plus IGF-I than in cultures treated with IGF-I alone (Figure 3). In two additional experiments comparing 200 vs 400 ng/mL of IGF-I in the absence of FSH, 400 ng/mL of IGF-I increased (*P* < .05) progesterone production above that achieved with 200 ng/mL (12.5 vs 8.0 ± 2.3 ng·10<sup>5</sup> cells<sup>-1</sup>·24 h<sup>-1</sup>).

**Effect of Follicle-Stimulating Hormone and Testosterone on Granulosa Cells from Small Follicles.** To investigate the effect of testosterone and FSH on granulosa cells, cells from small follicles were cultured for 2 d in 10% FCS followed by 2 d in serum-free medium containing insulin (5 μg/mL) alone, FSH (100 to 800 ng/mL) plus insulin, or testosterone (.1 to 3 μg/mL) plus insulin. Follicle-stimulating hormone did not affect cell numbers at d 4 of culture (data not

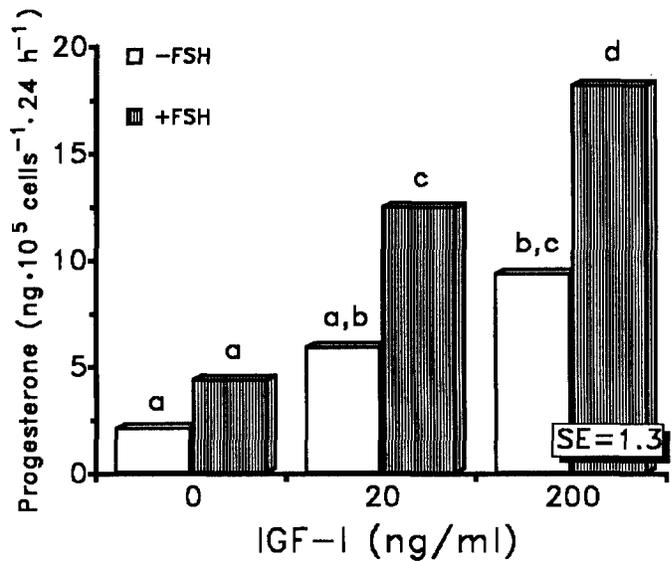


Figure 3. Effect of insulin-like growth factor I (IGF-I) in the absence or presence of follicle-stimulating hormone (FSH) on progesterone production on d 4 of culture. Granulosa cells from small follicles were cultured as described in legend for Figure 1. The second 2 d of culture included IGF-I (0, 20, or 200 ng/mL) and/or FSH (200 ng/mL). Medium was changed every 24 h. Progesterone concentrations in medium and cell numbers were determined on d 4. Values are least squares means of quadruplicate culture wells from two separate experiments. Bars without a common letter differ (*P* < .05). Pooled SE = 1.3 ng · 10<sup>5</sup> cells<sup>-1</sup> · 24 h<sup>-1</sup>.

shown). In contrast, testosterone (3 μg/mL) decreased cell numbers by 32% but did not affect progesterone production (nanograms·10<sup>5</sup> cells<sup>-1</sup>·24 h<sup>-1</sup>) in insulin-supplemented, serum-free medium on d 4 of culture (Table 1). Follicle-stimulating hormone (100, 200, 400, and 800 ng/mL) increased (*P* < .05) progesterone production at d 4 by 1.6-fold (13.2 vs 21.7 ± .8 ng·10<sup>5</sup> cells<sup>-1</sup>·24 h<sup>-1</sup> for control vs all FSH treatments, respectively); the levels of progesterone production stimulated by 100, 200, 400, and 800 ng/mL of FSH were similar (*P* > .10).

*Effect of Insulin and Insulin-Like Growth Factor I on Estradiol Production*

**Effects on Granulosa Cells from Large Follicles.** To determine the dose response of insulin and IGF-I on estradiol production by differentiated granulosa cells, cells from large follicles were cultured for 2 d in 10% FCS followed by 1 d in serum-free medium containing testosterone (3 μg/mL) and FSH (200 ng/mL) in the presence or absence of insulin (0, .1, 1, 5, or 10 μg/mL) or IGF-I (0, 100, or 200 ng/mL). In cultures of cells from large follicles (in serum-free medium containing only testosterone), insulin had no significant effect on estradiol production in the absence of

Table 1. Effects of testosterone on insulin-stimulated cell proliferation and progesterone production at day 4 of culture<sup>a</sup>

Dose of testosterone, $\mu\text{g}/\text{mL}$	Cell no. per well, $\times 10^{-5}$	Progesterone <sup>b</sup> , $\text{ng} \cdot 10^5 \text{ cells}^{-1} \cdot 24 \text{ h}^{-1}$
0	.96 <sup>d</sup>	22.4 <sup>c</sup>
1.0	.92 <sup>d</sup>	20.9 <sup>c</sup>
3.0	.65 <sup>c</sup>	21.7 <sup>c</sup>
Pooled SE	.08	2.5

<sup>a</sup>Means are least squares means from two separate experiments in which treatments were applied in quadruplicate cultures. Insulin was applied at 5  $\mu\text{g}/\text{mL}$ .

<sup>b</sup>Mean secretion from d 3 to 4 of culture corrected for cell numbers on d 4.

<sup>c,d</sup>Means within a column with different superscripts differ ( $P < .05$ ).

FSH (Spicer et al., 1992a), and thus all cell cultures were treated with FSH (200 ng/mL). In the presence of FSH, IGF-I had a biphasic dose-dependent effect on estradiol production; 100 ng/mL, but not 200 ng/mL, of

IGF-I increased ( $P < .05$ ) estradiol production by 35% (Figure 4A). Similarly,  $\geq 5 \mu\text{g}/\text{mL}$  of insulin had no significant effect on estradiol production, whereas 1  $\mu\text{g}/\text{mL}$  increased ( $P < .05$ ) estradiol production by 37% (Figure 4B). In comparison, IGF-II (1 to 100 ng/mL) had no effect ( $P > .05$ ) on estradiol production by granulosa cells from large follicles (Table 2).

*Effects on Granulosa Cells from Small Follicles in the Absence of Follicle-Stimulating Hormone.* To determine the effect of insulin and IGF-I on estradiol production by undifferentiated granulosa cells, cells from small follicles were cultured for 2 d in 10% FCS followed by 1 d in serum-free medium containing 3  $\mu\text{g}/\text{mL}$  of testosterone in the absence of FSH with or without insulin (0, .1, 1, or 5  $\mu\text{g}/\text{mL}$ ) or IGF-I (0, 100, or 200 ng/mL). At doses of .1 and 1.0  $\mu\text{g}/\text{mL}$ , insulin increased ( $P < .05$ ) estradiol production by 58 and 72%, respectively (Figure 5B). However, 5.0  $\mu\text{g}/\text{mL}$  of insulin had no significant effect on estradiol production. In contrast, 200 ng/mL of IGF-I did not affect ( $P > .10$ ) estradiol production, whereas 100 ng/mL inhibited ( $P < .05$ ) estradiol production by 28% (Figure 5A).

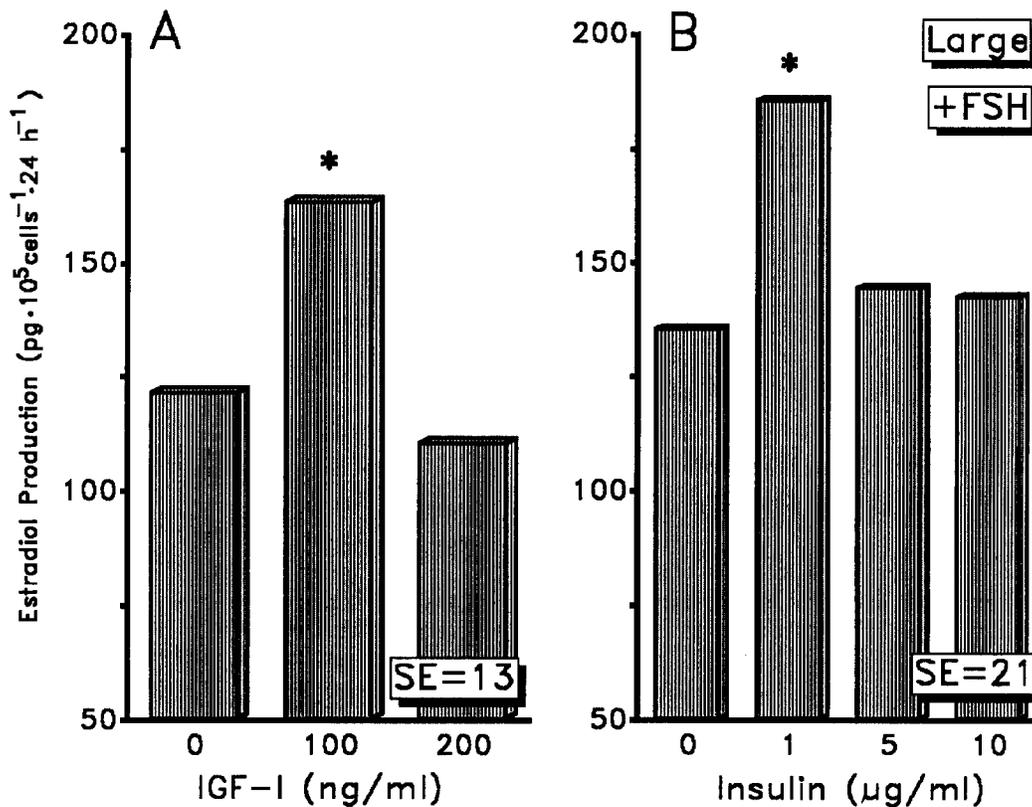


Figure 4. Dose-response of insulin-like growth factor I (IGF-I) (Panel A) and insulin (Panel B) on estradiol production by granulosa cells from large follicles. Cells were cultured for 2 d in the presence of 10% fetal calf serum and then treated with various doses of insulin or IGF-I in the presence of 200 ng/mL of follicle-stimulating hormone (FSH) and 3  $\mu\text{g}/\text{mL}$  of testosterone for an additional 24 h. Values are means of quadruplicate culture wells from three separate experiments for Panel A and two separate experiments for Panel B. Pooled SE = 13 and 21  $\text{pg} \cdot 10^5 \text{ cells}^{-1} \cdot 24 \text{ h}^{-1}$  for Panels A and B, respectively. \*Mean differs from Control (0 ng/mL or 0  $\mu\text{g}/\text{mL}$ ).

Table 2. Dose-response of insulin-like growth factor II (IGF-II) on proliferation and estradiol production by granulosa cells cultured from large ( $\geq 8$  mm) follicles in the presence of follicle-stimulating hormone<sup>a</sup>

Dose of IGF-II, ng/mL	Cell no. per well, $\times 10^{-5}$	Estradiol production, $\text{pg}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$
0	.11	110
1	.13	120
10	.16	91
20	.13	90
50	.13	109
100	.14	104
Pooled SE	.01	10

<sup>a</sup>Means are least squares means of quadruplicate culture wells from two separate experiments.

*Effects on Granulosa Cells from Small Follicles in the Presence of Follicle-Stimulating Hormone.* To determine whether FSH altered the response of granulosa cells to IGF-I, cells from small follicles were treated with FSH (200 ng/mL) and testosterone (3

$\mu\text{g/mL}$ ) in the presence or absence of IGF-I (0, 100, or 200 ng/mL); both 100 and 200 ng/mL of IGF-I inhibited ( $P < .05$ ) estradiol production by 28 to 46% ( $71.4, 51.4, \text{ and } 38.5 \pm 9.1 \text{ pg}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$  for FSH alone, FSH plus 100 ng/mL of IGF-I, and FSH plus 200 ng/mL of IGF-I, respectively;  $n = \text{two experiments}$ ). In comparison, 100 ng/mL of IGF-II had no effect ( $P > .05$ ) on estradiol production by granulosa from small follicles ( $24.1 \text{ and } 28.1 \pm 9.8 \text{ pg}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$  for FSH alone and FSH plus IGF-II, respectively;  $n = \text{two experiments}$ ).

*Insulin-Like Growth Factor I mRNA Levels in Granulosa Cells*

To determine whether IGF-I is produced by granulosa cells, Northern blot analyses were conducted to assess IGF-I mRNA levels in granulosa cells collected and pooled from six FSH-treated cows. Liver tissue was also collected and pooled for comparison. Tissue was processed as described in the Materials and Methods section. As shown in Figure 6, multiple IGF-I mRNA transcripts were detected in both granulosa cells and liver tissue with major bands at

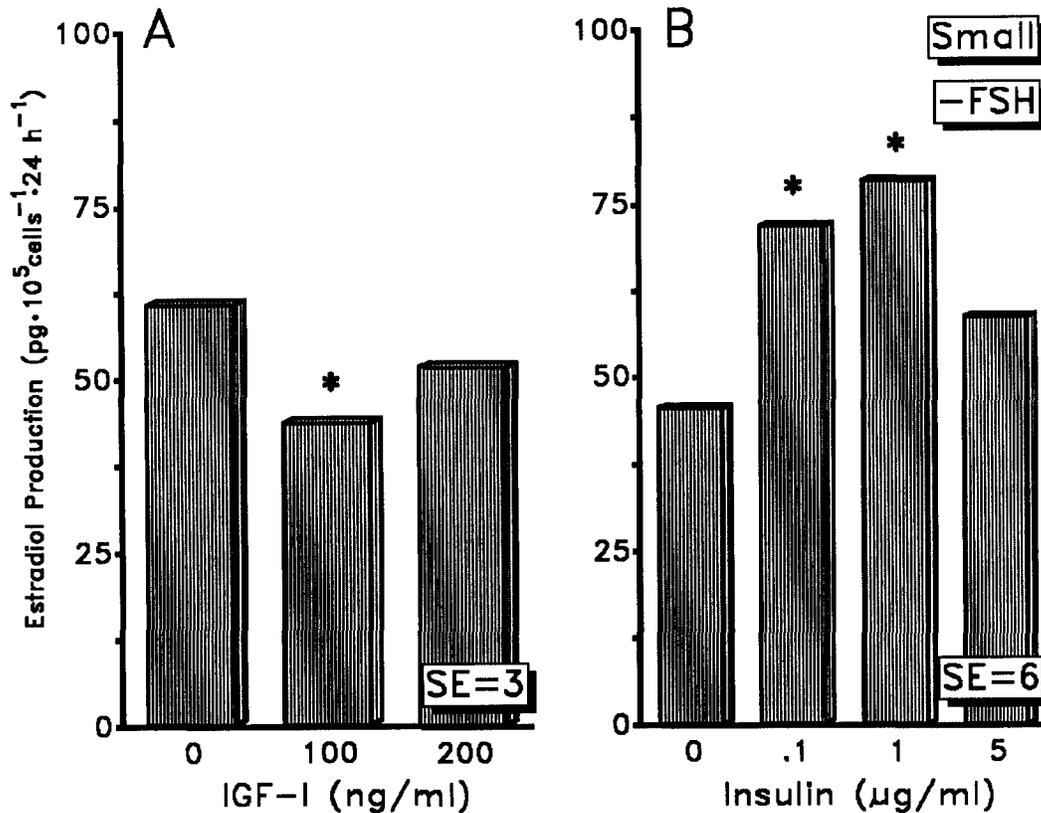


Figure 5. Dose-response of insulin-like growth factor I (IGF-I) (Panel A) and insulin (Panel B) on estradiol production by granulosa cells from small follicles. Cells were cultured as described in legend for Figure 4 except that no follicle-stimulating hormone (FSH) was given during the last 24 h of culture. Values are means of quadruplicate culture wells from two separate experiments. Pooled SE = 3 and 6  $\text{pg}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$  for Panels A and B, respectively. \*Mean differs ( $P < .05$ ) from Control (0 ng/mL or 0  $\mu\text{g/mL}$ ).

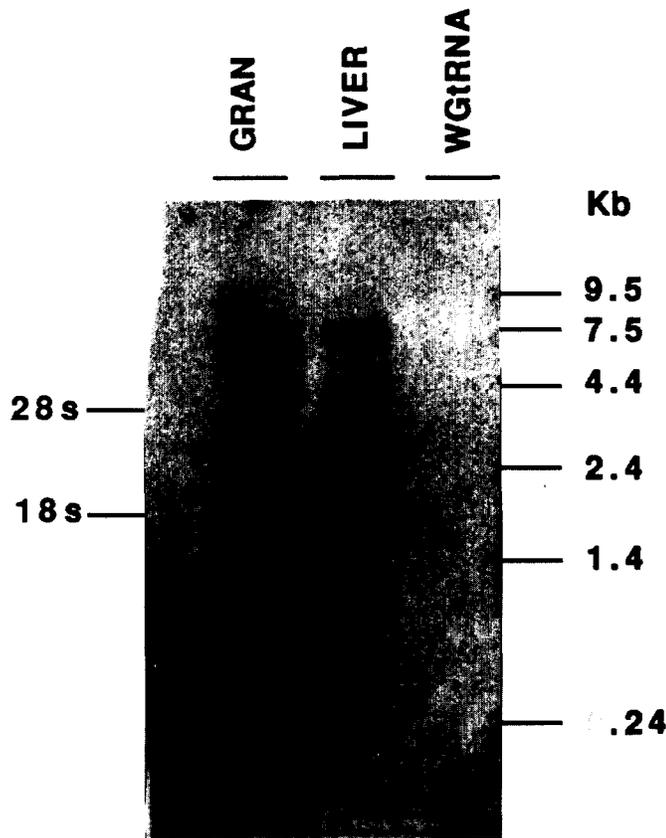


Figure 6. Expression of insulin-like growth factor I (IGF-I) mRNA in isolated granulosa cells (GRAN) and liver tissue pooled from six follicle-stimulating hormone-treated cows. Liver and granulosa cell RNA was subjected to electrophoresis, transferred to nitrocellulose filters, and hybridized with  $^{32}\text{P}$ -labeled IGF-I cDNA. Each lane contained an equal amount of total cellular RNA (40  $\mu\text{g}/\text{lane}$ ). The third lane contains a wheat germ RNA (WGT RNA) as a control. kb = kilobases.

7.0 to 7.5, 3.6 to 3.8, 1.8 to 1.9, and .7 to .9 kilobases (kb) with similar apparent abundance for the two tissues. Variability among individual animals was evaluated in tissue samples from four cows that were treated with FSH. As seen with the pooled samples, multiple IGF-I mRNA transcripts were detected in both granulosa cells and liver tissue with major bands at 7.0 to 7.5, 3.6 to 3.8, 1.8 to 1.9, and .7 to .9 kb (Figure 7). Interestingly, two of four cows contained more IGF-I mRNA in liver tissue than in granulosa cells, whereas the remaining two cows contained more IGF-I mRNA in granulosa cells than in liver tissue (Figure 7).

#### Regulation of Insulin-Like Growth Factor I Production In Vitro

To determine whether granulosa cell IGF-I production is hormonally regulated, cells from small follicles

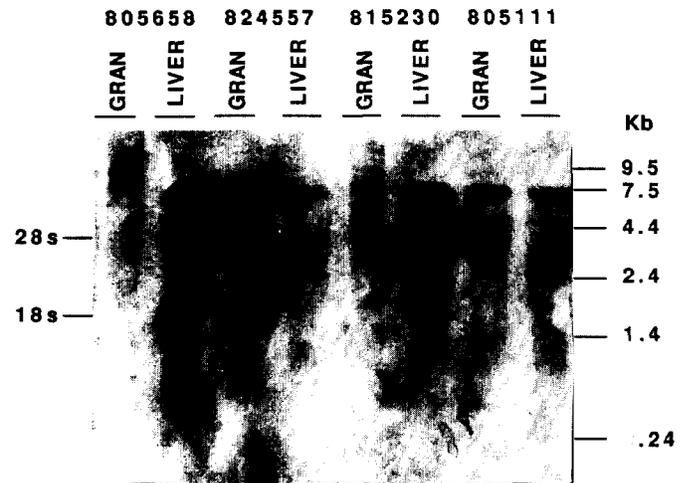


Figure 7. Expression of insulin-like growth factor I (IGF-I) mRNA in isolated granulosa cells (GRAN) and liver tissue collected from four individual cows. Liver and granulosa cell RNA was collected from follicle-stimulating hormone-treated cows and processed as described in Figure 6. kb = kilobases.

were cultured for 2 d in 10% FCS followed by 2 d in serum-free medium containing control (no additions), GH, insulin, EGF, and/or FSH. Table 3 shows that GH (300 ng/mL) or insulin (5  $\mu\text{g}/\text{mL}$ ) alone had no effect on, whereas insulin plus GH tended to decrease ( $P < .10$ ), IGF-I production in vitro. In additional experiments, FSH (200 ng/mL) had no significant effect on in vitro production of IGF-I in the presence of 5  $\mu\text{g}/\text{mL}$  of insulin (1.23 and 1.07  $\text{ng}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$  for insulin alone and FSH plus insulin, respectively;  $n =$  three experiments). Also, EGF (10 ng/mL) had no effect ( $P > .20$ ) on IGF-I production in the presence of 5  $\mu\text{g}/\text{mL}$  of insulin (1.34 and .93  $\text{ng}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$  for insulin and EGF plus insulin, respectively;  $n =$  two experiments).

Table 3. Hormonal regulation of insulin-like growth factor I (IGF-I) production in cultured granulosa cells<sup>a</sup>

Hormone	IGF-I production <sup>b</sup> , $\text{ng}\cdot 10^5 \text{ cells}^{-1}\cdot 24 \text{ h}^{-1}$
Control	2.18 <sup>cd</sup>
Insulin (5 $\mu\text{g}/\text{mL}$ )	1.32 <sup>cd</sup>
GH (300 ng/mL)	2.46 <sup>c</sup>
Insulin + GH	.75 <sup>d</sup>
Pooled SE	1.16

<sup>a</sup>Means are least squares means from three separate experiments.

<sup>b</sup>Mean IGF-I secretion from d 3 to 4 of culture corrected for cell numbers on d 4.

<sup>c,d</sup>Means within a column with no common superscripts differ ( $P < .10$ ). An insulin effect existed ( $P < .10$ ).

## Discussion

Insulin and IGF-I increased cell proliferation and progesterone production by bovine granulosa cells from small follicles in the present study. This is in agreement with the results of others who have found that insulin and IGF-I increase proliferation of bovine (Savion et al., 1981) and porcine (Baranao and Hammond, 1985; May et al., 1988) granulosa cells cultured in serum-free medium. Numerous reports also have shown beneficial effects of insulin and(or) IGF-I on bovine (Savion et al., 1981; Schams et al., 1988; McArdle, 1990), ovine (Monniaux and Pisselet, 1992), and porcine (Veldhuis et al., 1985; Maruo et al., 1988) granulosa cell progesterone production *in vitro*. In addition, we observed an FSH-induced increase in progesterone production by bovine granulosa cells treated with insulin and IGF-I; this observation is similar to previous reports using rat (Davoren and Hsueh, 1984; Adashi et al., 1985) and porcine granulosa cells (May and Schomberg, 1981; Baranao and Hammond, 1985; Gooneratne et al., 1990). Collectively, these results indicate that insulin and IGF-I enhance FSH-induced progesterone production of granulosa cells regardless of species.

In agreement with a previous study using porcine granulosa cells (Spicer and Hammond, 1989), we observed an inhibitory effect of androgens on bovine granulosa cell proliferation. Also similar to a previous study using bovine granulosa cells (Henderson and Franchimont, 1983), testosterone had no effect on progesterone production in the present study. This is in contrast with other studies that have shown an enhancing effect of androgens on progesterone production in porcine granulosa cells cultured in platelet extract-containing medium (Spicer and Hammond, 1988) and in bovine granulosa cells cultured in medium containing 10% calf serum (Henderson et al., 1987), suggesting that methodological differences (i.e., presence of serum factors) and(or) species differences may influence androgen regulation of granulosa cell steroidogenesis.

Insulin and IGF-I seem to augment FSH-stimulated estrogen production by cultured rat granulosa cells (Davoren and Hsueh, 1984; Adashi et al., 1985). In cultured porcine granulosa cells, insulin alone has either no effect (Maruo et al., 1988) or inhibitory effects (Veldhuis et al., 1983) on estradiol production, whereas IGF-I has only stimulatory effects on estradiol production (Veldhuis and Demers, 1985; Maruo et al., 1988). We observed that both insulin and IGF-I could stimulate estradiol production by bovine granulosa cells, depending on the dose, size of follicle, and the presence of FSH. Insulin was more effective at stimulating estradiol production by granulosa cells from small vs large follicles, whereas IGF-I was more effective at stimulating estradiol production by granulosa cells from large vs small

follicles. Thus, differences in hormone concentrations, size of follicle, and(or) presence of gonadotropins in medium could explain why studies evaluating the regulation of estradiol production in cultured porcine granulosa cells show variable results. As with previous studies (Anderson et al., 1979; Haney and Schomberg, 1981), granulosa cells from large follicles had a greater capacity to produce estradiol than did cells from small follicles.

Production of IGF-I by granulosa cells seems to be hormonally regulated in a manner different from that observed in hepatocytes. Previous studies have shown that GH and insulin are stimulatory to IGF-I production by rat hepatocytes *in vitro* (Daughaday et al., 1976; Schalch et al., 1979; Kachra et al., 1991). In the present study, insulin tended to inhibit IGF-I production, particularly in the presence of GH, and IGF-I production was unaffected by FSH and EGF. Further support of the suggestion that IGF-I production by liver and granulosa cells may be differentially regulated is obtained from our observations that during short periods of feed withdrawal in cattle IGF-I concentrations in blood decrease, whereas IGF-I concentrations in follicular fluid remain unchanged (Spicer et al., 1992b). Moreover, GH injections for 5 d in hypophysectomized rats resulted in a fivefold increase in hepatic IGF-I mRNA levels with a slight decrease in ovarian IGF-I mRNA (Hernandez et al., 1989). Similar to our present study, studies using cultured porcine granulosa cells (Mondschein and Hammond, 1988; Bryan et al., 1991) have not indicated an effect of EGF on basal IGF-I production in long-term (> 3 d) culture. However, in cultures treated with FSH plus estradiol, EGF stimulated IGF-I production by porcine granulosa cells in short-term (3 d) culture (Mondschein and Hammond, 1988; Bryan et al., 1991). In contrast to the present study, GH and FSH alone stimulated IGF-I production by porcine granulosa cells treated with insulin and cortisol in long-term culture (Hsu and Hammond, 1987a,b; Bryan et al., 1991). Thus, species and(or) methodological differences may exist in terms of hormone-regulated IGF-I production by granulosa cells.

Results from the present study using Northern blotting and hybridization demonstrated that the human IGF-I cDNA used binds to mRNA of adult bovine liver and granulosa cells; transcripts of approximately 7 to 8 kb, 4 kb, 2 kb, and 1 kb were found in liver tissue and granulosa cells. Similar-sized transcripts have been identified in adult bovine (Godfredson et al., 1991; Hannon et al., 1991) and human (Rotwein, 1986) liver and in adult bovine endometrium (Geisert et al., 1991). Moreover, IGF-I mRNA has been detected in human granulosa cells (Voutilainen and Miller, 1987) and rat ovaries (Hernandez et al., 1989; Oliver et al., 1989). The function of the various IGF-I mRNA species, their

transcription, processing, and translation are unknown. Because the 7.5-kb IGF-I mRNA has a shorter half-life than the 1.2-kb mRNA (Hepler et al., 1990), perhaps the expression of various transcript sizes may affect the rate of translation and(or) stability.

Although the present and previous studies (Savion et al., 1981; Geisthovel et al., 1990; Hammond et al., 1991) establish granulosa cells as a site of insulin and IGF-I action, their physiologic relevance remains unclear. Average concentrations of insulin in blood of beef and dairy cattle are usually < 10 ng/mL (Elsasser et al., 1989; Richards et al., 1989), and thus fall below the effective doses of insulin used in the present study. However, IGF-I concentrations in blood and follicular fluid in cattle usually exceed 100 ng/mL (Echternkamp et al., 1990; Spicer and Enright, 1991; Spicer et al., 1992b). Thus, IGF-I may be a more physiologically relevant promoter of follicular function than insulin.

### Implications

Insulin and insulin-like growth factor I (IGF-I) stimulated proliferation of bovine granulosa cells in vitro. In addition, follicle-stimulating hormone, insulin, and IGF-I stimulated granulosa cell progesterone production and(or) estradiol production. These studies indicate that IGF-I (and perhaps insulin) may have direct local effects on bovine ovarian follicular function in vivo, and that these effects are influenced by the differentiative state of the follicle.

### Literature Cited

- Adashi, E. Y., C. E. Resnick, J. D'Ercole, M. E. Svoboda, and J. J. Van Wyk. 1985. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr. Rev.* 6:400.
- Adashi, E. Y., C. Resnick, E. R. Hernandez, M. E. Svoboda, E. Hoyt, D. R. Clemmons, P. K. Lund, and J. J. Van Wyk. 1989. Rodent studies on the potential relevance of insulin-like growth factor (IGF-I) to ovarian physiology. In: A. N. Hirschfield (Ed.) *Growth Factors and the Ovary*. Plenum Press, New York.
- Anderson, L. D., F. W. Schaerf, and C. P. Channing. 1979. Effects of follicular development on the ability of cultured porcine granulosa cells to convert androgens to estrogen. *Adv. Exp. Med. Biol.* 112:187.
- Baranao, J.L.S., and J. M. Hammond. 1985. Serum-free medium enhances growth and differentiation of cultured pig granulosa cells. *Endocrinology* 116:51.
- Bryan, K. A., A. M. Clark, J. M. Hammond, and D. R. Hagen. 1991. Effect of constant versus adjusted dose of exogenous porcine growth hormone (pGH) on growth and reproductive characteristics of gilts. *J. Anim. Sci.* 69:2980.
- Bryan, K. A., J. M. Hammond, S. Canning, J. Mondschein, D. E. Carbaugh, A. M. Clark, and D. R. Hagen. 1989. Reproductive and growth responses of gilts to exogenous porcine pituitary growth hormone. *J. Anim. Sci.* 67:196.
- Burke, M. G., R. T. Stone, and N. E. Muggli-Crockett. 1991. Nucleotide sequence and Northern analysis of a bovine major histocompatibility class II DRB-like cDNA. *Anim. Genet.* 22:343.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
- Daughaday, W. H., L. S. Phillips, and M. C. Mueller. 1976. The effects of insulin and growth hormone on the release of somatomedin by the isolated rat liver. *Endocrinology* 98:1214.
- Davoren, J. B., and A.J.W. Hsueh. 1984. Insulin enhances FSH-stimulated steroidogenesis by cultured rat granulosa cells. *Mol. Cell. Endocrinol.* 35:97.
- Echternkamp, S. E., L. J. Spicer, K. E. Gregory, S. F. Canning, and J. M. Hammond. 1990. Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid in cattle selected for twins. *Biol. Reprod.* 43:8.
- Elsasser, T. H., T. S. Rumsey, and A. C. Hammond. 1989. Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-I in beef cattle. *J. Anim. Sci.* 67:128.
- Geisert, R. D., C.-Y. Lee, F. A. Simmen, M. T. Zavy, A. E. Fliss, F. W. Bazer, and R.C.M. Simmen. 1991. Expression of messenger RNAs encoding insulin-like growth factor-I, -II, and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. *Biol. Reprod.* 45:975.
- Geisthovel, F., I. Moretti-Rojas, F. J. Rojas, and R. H. Asch. 1990. Insulin-like growth factors and thecal-granulosa-cell function. *Human Reprod.* 5:785.
- Godfredson, J. A., M. D. Holland, K. G. Odde, and K. L. Hossner. 1991. Hypertrophy and hyperplasia of bovine fetal tissue during development: Fetal liver insulin-like growth factor I mRNA expression. *J. Anim. Sci.* 69:1074.
- Gooneratne, A. D., P. A. Thacker, B. Garveld, B. D. Murphy, and K. Rajkumar. 1990. Comparative effects of insulin and insulin-like growth factor-I on follicle-stimulating hormone-induced responses in porcine granulosa cells. *Steroids* 55:105.
- Hammond, J. M., J. S. Mondschein, S. E. Samaras, and S. F. Canning. 1991. The ovarian insulin-like growth factors, a local amplification mechanism for steroidogenesis and hormone action. *J. Steroid Biochem. Mol. Biol.* 40:411.
- Haney, A. F., and D. W. Schomberg. 1981. Estrogen and progesterone production by developing porcine follicles in vitro: Evidence for estrogen formation by theca. *Endocrinology* 109:971.
- Hannon, K., A. Gronowski, and A. Trenkle. 1991. Relationship of liver and skeletal muscle IGF-I mRNA to plasma GH profile, production of IGF-I by liver, plasma IGF-I concentrations, and growth rates of cattle. *Proc. Soc. Exp. Biol. Med.* 196:155.
- Henderson, K. M., and P. Franchimont. 1983. Inhibin production by bovine ovarian tissues in vitro and its regulation by androgens. *J. Reprod. Fertil.* 67:291.
- Henderson, K. M., K. P. McNatty, P. Smith, M. Gibb, L. E. O'Keefe, S. Lun, D. A. Heath, and M. D. Prisk. 1987. Influence of follicular health on the steroidogenic and morphological characteristics of bovine granulosa cells in vitro. *J. Reprod. Fertil.* 79:185.
- Hepler, J. E., J. J. Van Wyk, and P. K. Lund. 1990. Different half-lives of insulin-like growth factor-I mRNAs that differ in length of 3'-untranslated sequence. *Endocrinology* 127:1550.
- Hernandez, E. R., C. T. Roberts, Jr., D. LeRoith, and E. Y. Adashi. 1989. Rat ovarian insulin-like growth factor-I (IGF-I) gene expression is granulosa cell-selective: 5'-untranslated mRNA variant representation and hormonal regulation. *Endocrinology* 125:572.
- Hsu, C. J., and J. M. Hammond. 1987a. Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells in vitro. *Endocrinology* 120:198.
- Hsu, C. J., and J. M. Hammond. 1987b. Concomitant effects of growth hormone on secretion of insulin-like growth factor I and progesterone by cultured porcine granulosa cells. *Endocrinology* 121:1343.
- Kachra, Z., I. Barash, C. Yannopoulos, M. N. Khan, H. J. Guyda, and B. I. Posner. 1991. The differential regulation by glucagon and growth hormone of insulin-like growth factor (IGF)-I and

- IGF binding proteins in cultured rat hepatocytes. *Endocrinology* 128:1723.
- Langhout, D. J., L. J. Spicer, and R. D. Geisert. 1991. Development of a culture system for bovine granulosa cells: Effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *J. Anim. Sci.* 69:3321.
- Maruo, T., M. Hayashi, H. Matsuo, Y. Ueda, H. Morikawa, and M. Mochizuki. 1988. Comparison of the facilitative roles of insulin and insulin-like growth factor I in the functional differentiation of granulosa cells: In vitro studies with the porcine model. *Acta Endocrinol. (Kopenh.)* 117:230.
- May, J. V., J. P. Frost, and D. W. Schomberg. 1988. Differential effects of epidermal growth factor, somatomedin-C/insulin-like growth factor I, and transforming growth factor- $\beta$  on porcine granulosa cell deoxyribonucleic acid synthesis and cell proliferation. *Endocrinology* 123:168.
- May, J. V., and D. W. Schomberg. 1981. Granulosa cell differentiation in vitro: Effect of insulin on growth and functional integrity. *Biol. Reprod.* 25:421.
- McArdle, C. A. 1990. Chronic regulation of ovarian oxytocin and progesterone release by prostaglandins: Opposite effects in bovine granulosa and early luteal cells. *J. Endocrinol.* 126:245.
- Mondschein, J. S., and J. M. Hammond. 1988. Growth factors regulate immunoreactive insulin-like growth factor-I production by cultured porcine granulosa cells. *Endocrinology* 123:463.
- Monniaux, D., and C. Pisselet. 1992. Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro. *Biol. Reprod.* 46:109.
- Oliver, J. E., T. J. Aitman, J. F. Powell, C. A. Wilson, and R. N. Clayton. 1989. Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. *Endocrinology* 124:2671.
- Ott, L. 1977. *An Introduction to Statistical Methods and Data Analysis.* p 384. Duxbury Press, North Scituate, MA.
- Richards, M. W., R. P. Wettemann, and H. M. Schoenemann. 1989. Nutritional anestrus in beef cows: Concentrations of glucose and nonesterified fatty acids in plasma and insulin in serum. *J. Anim. Sci.* 67:2354.
- Rotwein, P. 1986. Two insulin-like growth factor I messenger RNAs are expressed in human liver. *Proc. Natl. Acad. Sci. USA.* 83:77.
- SAS. 1988. *SAS/STAT® User's Guide: Statistics.* SAS Inst. Inc., Cary, NC.
- Savion, N., G. Lui, R. Laherty, and D. Gospodarowicz. 1981. Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* 109:409.
- Schalch, D. S., U. E. Heinrich, D. Draznin, C. J. Johnson, and L. L. Miller. 1979. Role of the liver in regulating somatomedin activity: Hormonal effects on the synthesis and release of insulin-like growth factor and its carrier protein by the isolated perfused rat liver. *Endocrinology* 104:1143.
- Schams, D., R. Koll, and C. H. Li. 1988. Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. *J. Endocrinol.* 116:97.
- Spicer, L. J., E. Alpizar, and E. C. Short, Jr. 1992a. Regulation of aromatase activity in cultured bovine granulosa cells: Effects of cytokines are dependent on size of follicle. p 57. In: *Proc. IX Ovarian Workshop on Ovarian Cell Interactions: Genes to Physiology.* July 9-11, 1992. Chapel Hill, NC.
- Spicer, L. J., M. A. Crowe, D. J. Prendiville, D. Goulding, and W. J. Enright. 1992b. Systemic but not intraovarian concentrations of insulin-like growth factor-I are affected by short-term fasting. *Biol. Reprod.* 46:920.
- Spicer, L. J., S. E. Echterkamp, S. F. Canning, and J. M. Hammond. 1988. Relationship between concentrations of immunoreactive insulin-like growth factor-I in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biol. Reprod.* 39:573.
- Spicer, L. J., and W. J. Enright. 1991. Concentrations of insulin-like growth factor I and steroids in follicular fluid of preovulatory bovine ovarian follicles: Effect of daily injections of a growth hormone-releasing factor analog and (or) thyrotropin-releasing hormone. *J. Anim. Sci.* 69:1133.
- Spicer, L. J., and J. M. Hammond. 1988. Comparative effects of androgens and catecholestrogens on progesterone production by porcine granulosa cells. *Mol. Cell. Endocrinol.* 56:211.
- Spicer, L. J., and J. M. Hammond. 1989. Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells in vitro: Comparison with estradiol, 5 $\alpha$ -dihydrotestosterone, gonadotropins and catecholamines. *Mol. Cell. Endocrinol.* 64:119.
- Veldhuis, J. D., and L. M. Demers. 1985. A role for somatomedin C as a differentiating hormone and amplifier of hormone action on ovarian cells: Studies with synthetically pure human somatomedin C in swine granulosa cells. *Biochem. Biophys. Res. Commun.* 130:234.
- Veldhuis, J. D., R. W. Furlanetto, D. Juchter, J. Garmey, and P. Veldhuis. 1985. Trophic actions of human somatomedin C/insulin-like growth factor I on ovarian cells: In vitro studies with swine granulosa cells. *Endocrinology* 116:1235.
- Veldhuis, J. D., L. A. Kolp, M. E. Toaff, J. F. Strauss, III, and L. M. Demers. 1983. Mechanisms subserving the trophic actions of insulin on ovarian cells: In vitro studies using swine granulosa cells. *J. Clin. Invest.* 72:1046.
- Voutilainen, R., and W. L. Miller. 1987. Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side chain-cleavage enzyme, P450ssc, in human steroidogenic tissues. *Proc. Natl. Acad. Sci. USA* 84:1590.