Endocrine, *vol. 12, no. 1, 53–59, February 2000* 0969–711X/00/12/1:53–59/$11.75

Ovarian Action of Leptin

Effects on Insulin-Like Growth Factor-I–Stimulated Function of Granulosa and Thecal Cells

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To test the hypothesis that leptin signals metabolic information to the reproductive system in cattle by directly affecting IGF-I-induced ovarian cell function, granulosa and thecal cells from bovine ovarian follicles were cultured for 2 d in serum-free medium with added hormones. Recombinant human leptin at 30 and 300 ng/mL had no effect on basal thecal cell steroidogenesis or thecal cell numbers. However, 300 but not 30 ng/mL of leptin attenuated ($p < 0.05$) luteinizing hormone–induced androstenedione production by 24% in the absence of IGF-I and by 16% in the presence of IGF-I. Leptin had no effect on IGF-I-induced estradiol production in the presence of follicle-stimulating hormone (FSH), but at 100 ng/mL, leptin inhibited ($p < 0.05$) FSH plus IGF-I-induced progesterone production and granulosa cell proliferation by 29 and 31%, respectively. Leptin did not compete for ^{125}I-IGF-I binding to granulosa or thecal cells, whereas unlabeled IGF-I did. In conclusion, leptin has weak inhibitory effects on gonadotropin- and/or IGF-I-induced steroidogenesis of thecal and granulosa cells.

Key Words: Leptin; granulosa cells; thecal cells; steroidogenesis; cattle.

Introduction

Leptin, the recently identified adipose obese *(ob)* gene product, is a plasma protein hormone that parallels the amount of fat reserves in rodents and humans *(1–4)* and is thought to regulate satiety *(5–8)*. Whether systemic leptin concentrations correlate with body fat in cattle remains to be determined. The initial 167 amino acid sequence of leptin is highly conserved (i.e., 83–97% homology) as is the 145 amino acid sequence (the secreted form) of leptin (84–97% homology) across the four species (mouse, rat, human, monkey) in which it has been characterized *(9–13)*. If genetically obese *(ob/ob)* mice that lack endogenous leptin are injected with leptin, they experience decreased food intake, a loss in body weight, increased ovarian weight and number of follicles, and correction of a sterility defect *(5–8,14,15)*. In mature cows, reproductive performance decreases as body fat content decreases *(16)*. The latter observations indicate that leptin may have positive influences on the reproductive system in mice and cattle. However, recent in vitro evidence indicates that leptin may also have negative effects on ovarian cell function *(17–19)*.

Insulin-like growth factor-I (IGF-I) has long been known to have direct effects on ovarian cell function in several species including humans, rats, and cattle *(20,21)*. A decrease in systemic IGF-I concentrations is associated with the cessation of estrous cycles as cows are underfed to become nutritionally anestrus *(22)*. Conversely, increases in systemic IGF-I concentrations are associated with the resumption of estrous cycles as nutritionally anestrous cows and prepubertal heifers are refed to gain weight *(22,23)*. Thus, IGF-I may be one hormone, in addition to leptin, that communicates the metabolic status of an animal to the reproductive system in cattle. We hypothesized that leptin may directly influence IGF-I-stimulated ovarian follicular function in cattle and conducted experiments to determine the effect of leptin on IGF-I-induced proliferation and steroidogenesis of bovine granulosa and thecal cells in vitro.

Results

Experiment 1

Experiment 1 was conducted to evaluate the dose-response effect of leptin on basal and luteinizing hormone (LH)–induced thecal cell proliferation and/or steroidogenesis. Thecal cells from large (≥8 mm) follicles were cultured for 2 d in 10% fetal calf serum (FCS), and then cultured in serum-free medium for an additional 2 d with or without 100 ng/mL of LH in the presence of 0, 30, or 300 ng/mL of leptin. The doses of LH and leptin were selected based on previous studies *(17,18,24,25)*. In the absence of leptin, LH increased ($p < 0.05$) thecal cell progesterone (Fig. 1A) and androstenedione (Fig. 1B) production by 2.5- and 3.5-fold, respectively. By contrast, leptin had no effect ($p > 0.10$) on basal or LH-induced progesterone (Fig. 1A) or on basal androstenedione (Fig. 1B)

Received September 24, 1999; Revised November 27, 1999; Accepted December 2, 1999.

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production. The LH-induced increase in androstenedione production was reduced ($p < 0.05$; a 24% decrease) by 300 but not 30 ng/mL of leptin (Fig. 1). Leptin at 30 and 300 ng/mL had no effect ($p > 0.10$) on thecal cell numbers (Table 1).

Experiment 2

Experiment 2 was conducted to evaluate the effect of leptin on the dose response of thecal cells to LH in the presence of IGF-I. Thecal cells were cultured for 2 d in 10% FCS and then cultured in serum-free medium for an additional 2 d with 100 ng/mL of IGF-I in the absence or presence of 300 ng/mL of leptin and LH (0, 3, or 100 ng/mL). The doses of LH, IGF-I, and leptin were selected based on results from experiment 1 and on previous studies *(17,24–26)*. The effect of IGF-I in the absence of LH was not evaluated because previous studies showed that IGF-I had no effect on thecal steroidogenesis in the absence of LH *(24)*. In the absence of leptin but presence of IGF-I, LH at 3 and 100 ng/mL increased thecal cell ($p < 0.05$) progesterone production by 1.3- and 2.0-fold, respectively (Fig. 2A) and increased androstenedione production by 2.4- and 5.5-fold, respectively (Fig. 2B). Leptin inhibited ($p < 0.05$) the increase in androstenedione production (by 16%) (Fig. 2B) but not progesterone production (Fig. 2A) induced by 100 ng/mL of LH. Leptin had no effect ($p > 0.10$) on thecal cell numbers (Table 1).

Experiment 3A

Experiment 3 was conducted to evaluate the dose-response effect of leptin on basal and IGF-I-induced granulosa cell proliferation and/or steroidogenesis. Granulosa cells from small (1–5 mm; experiment 3A) or large (≥ 8mm; experiment 3B) follicles were cultured for 2 d in 10% FCS, and then cultured in serum-free medium for an additional 2 d with 50 ng/mL of FSH and 500 ng/mL of testosterone (as an estrogen precursor) with IGF-I (0 or 100 ng/mL) and leptin (0, 3, or 100 ng/mL). The doses of FSH, IGF-I, and leptin were selected based on results from previous studies *(18,27)*. In the absence of leptin but presence of FSH, IGF-I increased ($p < 0.001$) the production of small-follicle granulosa cell progesterone (Fig. 3A) by 2.6-fold and production of estradiol (Fig. 3B) by 3.6-fold. Leptin at 100 but not 3 ng/mL decreased ($p < 0.001$) FSH plus IGF-I-induced progesterone production by 29% in small-follicle granulosa cell cultures (Fig. 3A). Leptin had no effect ($P > 0.10$) on FSH plus IGF-I-induced estradiol production or on steroidogenesis in the presence of FSH alone (Fig. 3B). Leptin at 100 but not at 3 ng/mL decreased ($p < 0.05$) IGF-I-induced granulosa cell numbers by 31% (Table 1).

Experiment 3B

In the absence of leptin but presence of FSH, IGF-I increased ($p < 0.001$) the production of large-follicle granulosa cell progesterone (Fig. 4A) by 3.2-fold and production of estradiol (Fig. 4B) by 8.8-fold. Leptin had no effect ($p > 0.10$) on FSH plus IGF-I-induced progesterone and estradiol production or on steroidogenesis in the presence of FSH alone (Fig. 4). Leptin at 100 ng/mL but not 3 ng/mL decreased ($p < 0.05$) IGF-I-induced granulosa cell numbers by 21% (Table 1).

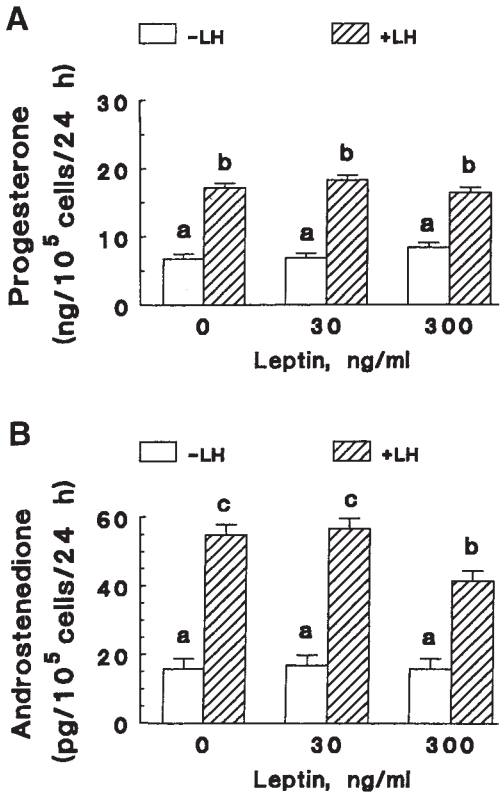

Fig.1. Effect of leptin on basal and LH-stimulated progesterone **(A)** and androstenedione **(B)** production by thecal cells (experiment 1). Thecal cells from large (≥8 mm) follicles were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media containing 0 (open bar) or 100 (hatched bars) ng/mL of LH for an additional 2 d. Medium was changed every 24 h. During the last 2 d of culture, leptin (0, 30, or 300 ng/mL) was also added to the medium. Values are means of four separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common letter (a,b,c) differ ($p < 0.05$).

Experiment 4

Experiment 4 was conducted to evaluate whether the inhibitory effect of leptin on thecal and granulosa cell function was owing to leptin directly inhibiting IGF-I binding to its receptors. Thecal and granulosa cells were cultured for 3 d in 10% FCS, the medium was removed, the cells were washed twice with 0.5 mL of 0.9% NaCl, and a ^{125}I-IGF-I binding assay was conducted for 16 h at 4°C as previously described *(24,28)*. Both 30 and 100 ng/well of IGF-I inhibited ($p < 0.05$) specific ^{125}I-IGF-I binding by granulosa (Fig. 5A) and thecal cells (Fig. 5B). By contrast, neither 30 nor 300 ng/well of leptin competed for specific granulosa and thecal cell ^{125}I-IGF-I binding (Fig. 5).

Table 1
Effect of Leptin on Basal and IGF-I-Induced Thecal (experiments 1 and 2) and Granulosa (experiments 3A and 3B) Cell Numbers

Dose of Leptin (ng/mL)	Dose of IGF-I (ng/mL)	Cell number (× 10^5 per well)[a,b]			
		Experiment 1	Experiment 2	Experiment 3A[c]	Experiment 3B[c]
0	0	1.94	—	0.20*	0.34*
3	0	—	—	0.25*	0.43*
30	0	1.95	—	—	—
100	0	—	—	0.21*	0.43*
300	0	1.95	—	—	—
0	100	—	3.31	1.27+	1.51+
3	100	—	—	1.28+	1.53+
100	100	—	—	0.87‡	1.19‡
300	100	—	3.35	—	—
SEM		0.04	0.07	0.10	0.07

[a]Because LH had no significant effect on cell numbers, values are means of all doses of LH combined for each dose of leptin and/or IGF-I.
[b]Within a column, means without a common symbol superscript differ ($p < 0.05$).
[c]All treatments were applied in the presence of 50 ng/mL of FSH and 500 ng/mL of testosterone.

Discussion

Results of the present studies indicate the following:

1. Leptin has no effect on basal or IGF-I-induced steroidogenesis of thecal cells but weakly inhibits LH-induced androstenedione production by thecal cells.
2. Leptin has no effect on basal and IGF-I-induced cell numbers of thecal cells.
3. Leptin has no effect on IGF-I-induced estradiol production by granulosa cells from small or large follicles.
4. Leptin at a high dose weakly inhibits IGF-I-induced cell numbers and progesterone production of granulosa cells from large and small follicles, respectively.
5. Leptin does not compete for ^{125}I-IGF-I binding in cultured thecal or granulosa cells.

Thus, these data do not support the hypothesis that leptin directly influences IGF-I action in bovine thecal cells but does support the hypothesis that leptin directly influences IGF-I action, albeit in a minor way, in bovine granulosa cells.

Similar to previous reports, we found that IGF-I increased thecal *(24,25)* and granulosa *(29,30)* cell numbers and steroidogenesis. However, results of the present study show, for the first time, that leptin has no influence on the stimulatory effect on IGF-I-induced thecal cell numbers and steroidogenesis. Leptin at a high dose (i.e., 300 ng/mL) weakly inhibited LH-induced androstenedione production but not progesterone production. Previously, we found that 10–300 ng/mL of recombinant mouse leptin inhibited LH plus insulin-induced progesterone and androstenedione production by >80% in cultured bovine thecal cells *(17)*. Because leptin attenuated LH-induced androstenedione production by <25% and had no effect on IGF-I-induced thecal cell steroid production in the present study, it seems likely that leptin's effect in bovine thecal cells is primarily directed toward insulin action. This latter conclusion likely holds true for bovine granulosa cells as well, because the inhibitory effect of leptin on FSH plus IGF-I-induced granulosa cell function in the present study was <30%, and in our previous studies *(18)* leptin inhibited insulin-induced granulosa cell steroidogenesis by 50–90%. However, additional studies in which the effects of leptin on IGF-I and insulin action are directly compared in the same experiment will be required to verify whether leptin's action on bovine thecal and granulosa cells are specific for insulin rather than IGF-I. Similarly, Brannian et al. *(31)* found that the inhibitory effect of leptin on human chorionic gonadtropin–stimulated progesterone production by human luteinized granulosa cells was only manifested in the presence of insulin. By contrast, leptin inhibited FSH plus IGF-I-induced estradiol production by <30% at optimal doses of FSH and by >80% at suboptimal doses of FSH in cultured rat granulosa cells *(19)*. Agarwal et al. *(32)* reported that leptin inhibited FSH plus IGF-I-induced estradiol production by human granulosa cells and LH plus IGF-I-induced androstenedione production by human thecal cells. Leptin has also been shown to inhibit LH-induced estradiol production by human granulosa cells cultured in the presence of 1% fetal bovine serum *(33)*, as well as dexamethasone-induced (but not FSH-induced) progesterone production by rat granulosa cells in serum-free medium *(34)*. Physiologically, the current and previous studies indicate that leptin at high concentrations (i.e., ≥100 ng/mL) that are found only in obese women *(2–4)* may alter insulin-, IGF-I, or gonadotropin-stimulated granulosa cell functions. Further studies will be required to elucidate the hormonal specificity of leptin's inhibitory action within the bovine, human, and rat ovary.

Note that the sequence of the 145 amino acid form of secreted leptin is highly conserved across species *(9–13)*. Specifically, bovine leptin (GenBank accession no. P50595)

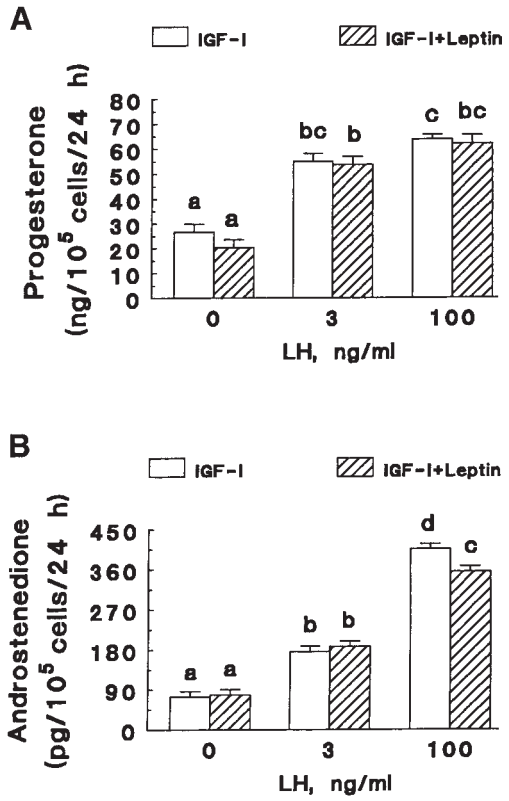

Fig. 2. Effect of leptin on IGF-I- and LH-stimulated progesterone **(A)** and androstenedione **(B)** production by thecal cells (experiment 2). Thecal cells from large (≥8 mm) follicles were cultured as described in Fig. 1. During the last 2 d of culture, LH (0, 3, or 100 ng/mL) was added to medium with 100 ng/mL of IGF-I and either 0 (open bar) or 300 ng/mL (hatched bar) of leptin. Values are means of three separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common letter (a,b,c,d) differ ($p < 0.05$).

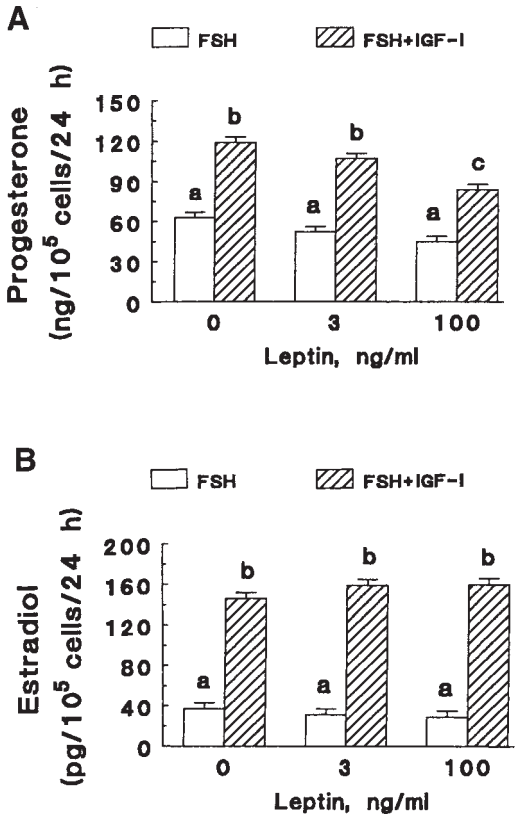

Fig. 3. Effect of leptin on FSH and FSH plus IGF-I-stimulated progesterone **(A)** and estradiol **(B)** production by granulosa cells from small follicles (experiment 3A). Granulosa cells from small (1–5 mm) follicles were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media containing leptin (0, 3, or 100 ng/mL), 50 ng/mL of FSH, 500 ng/mL of testosterone, and either 0 (open bar) or 100 ng/mL (hatched bar) of IGF-I for an additional 2 d. Medium was changed every 24 h. Values are means of three separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common letter (a,b,c,) differ ($p < 0.05$).

has 85% homology with mouse and rat leptin *(10,11,13)* and 86% homology with human leptin *(10,12)*. Thus, the effects of human leptin and bovine leptin on bovine ovarian cell function would likely yield similar results, but this awaits verification. Although the locus of leptin's action on thecal and granulosa cell function will also require additional studies, present and previous results indicate that the inhibitory effect of leptin on IGF-I action is not mediated by direct inhibition of IGF-I binding to its receptor (present study) or by direct inhibition of insulin binding to its receptor *(17,18)*. A recent report indicates that leptin can attenuate tyrosine phosphorylation of the insulin receptor substrate-1 in cultured HepG2 cells *(35)*, but whether this is the mechanism operative in granulosa or thecal cells remains to be determined. Additionally, recent studies indicate that injections of leptin to *ob*/*ob* mice, which have a congenital deficiency in leptin and are infertile, increase numbers of Graafian follicles *(14)* and correct their infertility *(15)*. The lack of a positive effect of leptin on granulosa and thecal cell proliferation observed in the present study is not consistent with these previous observations, as is the lack of effect of leptin on IGF-I-induced granulosa and thecal cell steroidogenesis. Furthermore, these in vitro effects of leptin observed in the present study are likely not owing to a change in cell viability because we have previously reported that insulin, leptin, and their combined treatment had no effect on cell viability *(17)*. Collectively, recent in vitro data indicate that leptin may exert a direct effect on ovarian function in vivo. Note, however, that, in vivo, leptin may have multiple sites of action within the hypothalamic-pituitary-ovarian axis and therefore positively or negatively impact other reproductive tissue. In support of this notion, serum LH and FSH concentrations were increased in *ob*/*ob* mice injected with leptin *(14)*, and the glutamatergic excitatory postsynaptic current in the arcuate nucleus was reduced with leptin treatment *(36)*.

Our previous studies have indicated that bovine ovarian granulosa and thecal cells have high-affinity receptors for

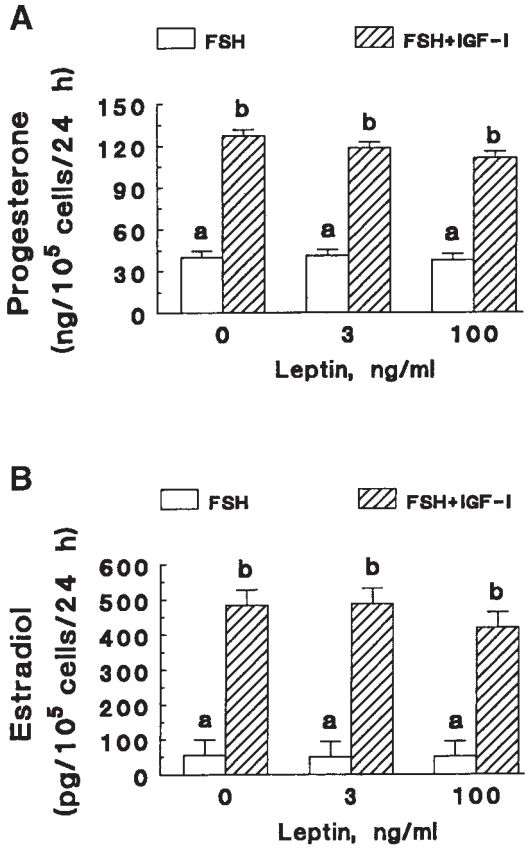

Fig. 4. Effect of leptin on IGF-I-stimulated progesterone **(A)** and estradiol **(B)** production by granulosa cells from large (≥8 mm) follicles (experiment 3B). Granulosa cells from large follicles were cultured as described in Fig. 3. During the last 2 d of culture, leptin (0, 3, or 100 ng/mL) was added to the medium with 50 ng/mL of FSH, 500 ng/mL of testosterone, and either 0 (open bar) or 100 ng/mL (hatched bar) of IGF-I. Values are means of three separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common letter (a,b) differ ($p < 0.05$).

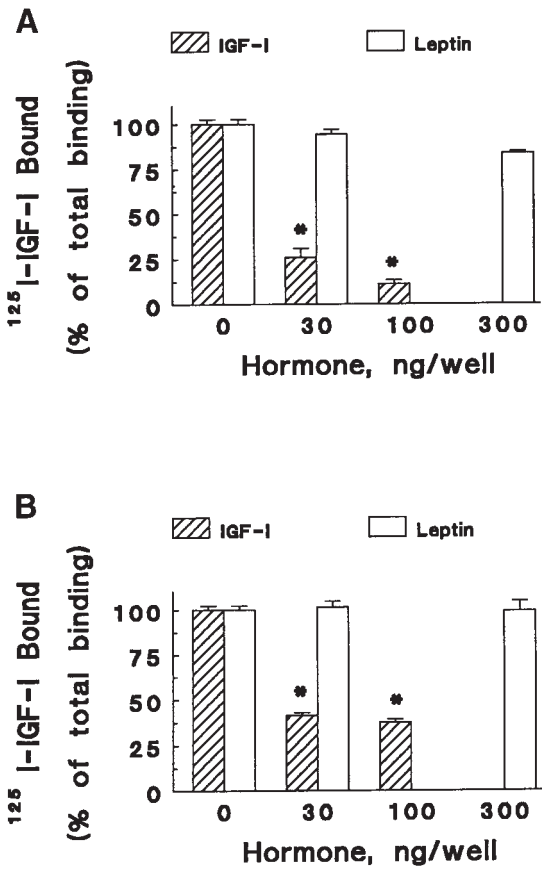

Fig. 5. Comparison of leptin and IGF-I on competing for ^{125}I-IGF-I binding by granulosa and thecal cells (experiment 4). Granulosa **(A)** and thecal **(B)** cells were cultured for 3 d in the presence of 10% FCS, and then cells were washed and IGF-I binding assays were conducted as described in Materials and Methods. Values are means of three separate experiments and are expressed as a percentage of total binding. Within each replicate experiment, each treatment was applied in triplicate culture wells. Asterisk indicates mean differs ($p < 0.05$) from control (0 ng/well).

leptin *(17,18)*. Comparison of the DNA sequence of the bovine leptin receptor *(37)* with that of human *(38)* and mouse *(39)* indicates an 81 and 75% sequence homology, respectively. This suggests that the leptin receptor, like leptin itself, may be noticeably conserved across species. Moreover, leptin receptor mRNA has been isolated from human granulosa and thecal cells *(32,33)* and rat ovary *(40)*. Therefore, the present and previous studies indicate that the bovine, human, and rat ovary are likely target organs for leptin.

In conclusion, the results of the present study indicate that leptin has only weak inhibitory effects on IGF-I-induced steroidogenesis and proliferation of granulosa cells. In thecal cells, leptin had weak inhibitory effects on LH-induced steroidogenesis with no effect on thecal cell proliferation. We suggest that if leptin plays a role in communicating the metabolic status of an animal to the reproductive system, it is not through a major alteration in the ovarian response to IGF-I, at least in thecal cells of cattle.

Materials and Methods

Reagents and Hormones

The following Reagents were used: Dulbecco's modified Eagle's medium (DMEM), Ham's F12, insulin (bovine; 25.7 U/mg), enzymes, and FCS, all obtained from Sigma (St. Louis, MO); bovine LH (L1914, LH activity 2.0 × NIH-LH-S1 U/mg) and ovine FSH (F1913, FSH activity 15 × NIH-FSH-51 U/mg), obtained from Scripps (San Diego, CA); recombinant human IGF-I, obtained from R&D Systems (Minneapolis, MN); and recombinant human leptin, obtained from Genentech (South San Francisco, CA).

Cell Culture

Ovaries were obtained at a nearby commercial abattoir from beef and dairy cattle after slaughter. After transport to the laboratory on ice (<120 min), the ovaries were processed, and granulosa and thecal cells were isolated as described

previously *(24,41)*. Briefly, granulosa cells from small (1–5 mm) and large (≥8 mm) follicles were collected by aspiration using a needle and syringe *(41)*. To isolate thecal cells, large (≥8 mm) follicles were dissected from the ovary, follicular fluid was aspirated, follicles were bisected, granulosa cells were removed, the theca interna layer was microdissected from the follicle wall, and the theca was enzymatically digested for 1 h at 37°C *(24)*. After incubation, undigested tissue was removed from the cell suspension by filtration and washed in serum-free medium. Contamination of thecal cells by granulosa cells is minimal using this procedure (<10%) *(27,42)*. Cells were resuspended in serum-free medium, and the number of viable cells was determined using the trypan blue exclusion method. Cell viability averaged 67 ± 10 and 94 ± 1% at the time of plating for granulosa and thecal cells, respectively.

The medium was a 1:1 (v/v) mixture of DMEM and Ham's F-12 containing 0.12 m*M* gentamicin and 38.5 m*M* sodium bicarbonate. Approximately 2×10^5 viable cells in 20–105 µL of medium was added to Falcon multiwell plates (#3047; Becton Dickinson, Lincoln Park, NJ) containing 1 mL of medium. Cultures were kept at 38.5°C in a 5% CO_2 atmosphere. To obtain optimal attachment, cells were maintained in the presence of 10% FCS for the first 2 d of culture. Next, cells were washed twice with 0.5 mL of serum-free medium, and incubations were continued in serum-free medium with or without added hormones unless stated otherwise. Medium was changed every day. For experiments evaluating the effects of hormones on steroid production, hormonal treatments were applied for 2 d (i.e., from d 2–4 of culture), unless stated otherwise. Under these culture conditions, we find that control cultures maintain cell numbers between d 1 and 2 of treatment *(24,41)*.

Determination of Cell Numbers

The numbers of granulosa and thecal cells were determined at the termination of experiments using a Coulter counter (Model Zm; Coulter Electronics, Hialeah, FL) as previously described *(24,41)*. Briefly, cells were exposed to 0.5 mL of trypsin (0.25% [w/v] in 0.15 *M* NaCl) for 20 min at 25°C, and then scraped from each well, diluted in 0.15 *M* NaCl and enumerated.

Androstenedione Radioimmunoassay

Concentrations of androstenedione in culture medium collected on day 4 of culture were determined using solid-phase radioimmunoassay (RIA) kits (ICN, Costa Mesa, CA) as previously described *(24)*. Intra- and interassay coefficients of variation (CVs) were 10 and 17%, respectively. Sensitivity of the androstenedione assay was 5 pg/tube.

Functional Aromatase Activity

Functional aromatase activity was assessed during a 24-h exposure of granulosa cells to 500 ng/mL of testosterone as previously described *(29,43)*. After the last 24-h incubation, concentrations of estradiol in medium were determined by RIA *(30)*. Intra- and interassay CVs were 9 and 12%, respectively. Sensitivity of the estradiol assay was .5 pg/tube.

Progesterone RIA

Concentrations of progesterone in culture medium collected on d 4 of culture were determined with a RIA as previously described *(41)*. Intra- and interassay CVs were 10 and 13%, respectively. Sensitivity of the progesterone assay was 25 pg/tube.

^{125}I-IGF-I Receptor Assay

Receptor assays for IGF-I were conducted as previously described *(24,28)*. Briefly, 50,000 dpm of ^{125}I-IGF-I was added directly into the 24-well culture plates alone, with unlabeled leptin or unlabeled IGF-I. The final assay volume was 500 µL of phosphate-buffered saline (PBS) (2.5% bovine serum albumin, pH 7.4). At the end of the 16-h incubation period at 4°C, the wells were washed with PBS, and the cells were solubilized with 1 *N* NaOH and placed in 12 × 75 mm tubes. Culture wells were washed twice, and these washes were combined with cells and counted in an automated gamma counter (counter efficiency = 75%).

Statistical Analyses

Experimental data are presented as least-squares means ± SEM of measurements *(44)* for triplicate culture wells from three or more experiments. For granulosa cells, each experiment was performed with different pools of cells collected from ovaries of 6–10 cows for each pool. For thecal cells, each experiment was performed with different pools of cells collected from five to eight follicles from four to seven cows for each pool. Main effects (e.g., dose) and interactions on dependent variables (steroid production) were assessed using the general linear models procedure of SAS *(44)*. Each well was a replicate, and each experiment contained three replicates per treatment. When steroid production was expressed as nanograms or picograms/(10^5 cells • 24 h), cell numbers at the termination of the experiment were used for this calculation. Specific differences in steroid production between treatments were determined using the Fisher's protected least significant difference procedure *(45)*.

Acknowledgments

We thank Genentech for donating the recombinant human leptin, Kent Kelly for technical assistance, Wellington Quality Meats (Wellington, KS) for donating of bovine ovaries, N.R. Mason (Lilly Research Laboratories) for donating estradiol antiserum, and Paula Cinnamon for secretarial assistance. This research was approved for publication by the Director, Oklahoma Agricultural Experiment Station, and supported under projects H-2088 and HR4-032 from the Oklahoma Center for Advancement of Science and Technology.

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