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Tumor Necrosis Factor-α (TNF-α) Inhibits Steroidogenesis of Bovine Ovarian Granulosa and Thecal Cells In Vitro

Involvement of TNF-α Receptors

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The effect of recombinant bovine tumor necrosis factor-α (TNF-α) on steroidogenesis and numbers of bovine ovarian granulosa and thecal cells has been studied, and specific binding sites for ^{125}I-TNF-α on ovarian cells have been determined. Granulosa cells have been examined from small (surface diameter 1–5 mm) follicles, whereas thecal cells from large (≥ 8 mm) follicles were utilized. Increasing doses of TNF-α significantly attenuated insulin- and IGF-I-induced estradiol production by granulosa cells from small follicles, but had no effect on basal estradiol production. Moreover, TNF-α significantly attenuated insulin- and LH-induced androstenedione production by thecal cells from large follicles. TNF-α had little or no effect on the numbers of granulosa and thecal cells in these same studies. Specific high-affinity, low-capacity binding of ^{125}I-TNF-α was also demonstrable in granulosa and thecal cells. Thus, it appears that TNF-α inhibits insulin- and IGF-I-induced estradiol production by granulosa cells and androstenedione production by thecal cells via TNF-α binding to its own receptor.

Key Words: Tumor necrosis factor-α; ovary; granulosa cells; thecal cells; steroidogenesis.

Introduction

Tumor necrosis factor-α (TNF-α) is a cytokine produced by activated marcophages *(1,2)* that is thought to play a major role in the cachexia and physiopathologic manifestation of shock produced by sepsis *(3–5)*. In cattle, systemic levels of TNF-α increase acutely during infection *(6,7)* and thus endocrine-like effects of TNF-α may be manifested. Indeed, TNF-α regulates function of endocrine cells, including ovarian granulosa cells and anterior pituitary cells *(8–10)*. In spite of the fact that TNF-α and other cytokines, such as interleukin-6 (IL-6), affect ovarian cell function *(8–12)*, the role of TNF-α in ovarian cell proliferation and steroidogenesis in cattle is not well characterized. The meager data available indicate that TNF-α inhibits follicle-stimulating hormone (FSH)-induced estradiol production by bovine granulosa cells *(13)*, but whether TNF-α alters insulin- or insulin-like growth factor-I (IGF-I)-induced granulosa or thecal cell function in cattle is unknown. In cattle, TNF-α-like activity is present in ovarian follicular fluid, and levels increase as follicles enlarge *(14)*. It is likely that some of the TNF-α in follicular fluid is derived from lymphocytes, including macrophages that infiltrate ovarian follicles and corpora lutea *(15–19)*. In bovine ovaries, TNF-α is localized in corpora lutea, in layers of granulosa cells lining the antral cavity of follicles, and throughout the granulosa layer of atretic follicles *(20)*. Therefore, the objectives of our studies were to evaluate further the effect of TNF-α on steroidogenesis and proliferation of bovine granulosa and thecal cells, and to determine if bovine granulosa and thecal cells contain specific binding sites for TNF-α.

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Results

Effect of TNF-α on Insulin-Induced Granulosa Cell Estradiol Production

The objective of these experiments was to evaluate the effect of TNF-α on insulin-induced estradiol production by granulosa cells of small follicles (Exp. 1). In the absence of TNF-α, insulin increased granulosa cell estradiol production by eightfold (Fig. 1). In the absence of insulin, basal estradiol (Fig. 1) production by granulosa cells from small follicles was not affected ($p > 0.10$) by TNF-α. In the presence of insulin, there was a significant inhibitory effect of TNF-α dosage on estradiol production by granulosa cells from small follicles (Fig. 1); at 30 ng/mL of TNF-α, estradiol production was 26% of control values. In the absence of TNF-α, insulin increased cell numbers by 2.3-fold above control values (Table 1). Basal cell numbers were not affected ($p > 0.10$) by TNF-α, but 30 ng/mL of TNF-α reduced ($p < 0.05$) insulin-stimulated cell numbers by 20% (Table 1).

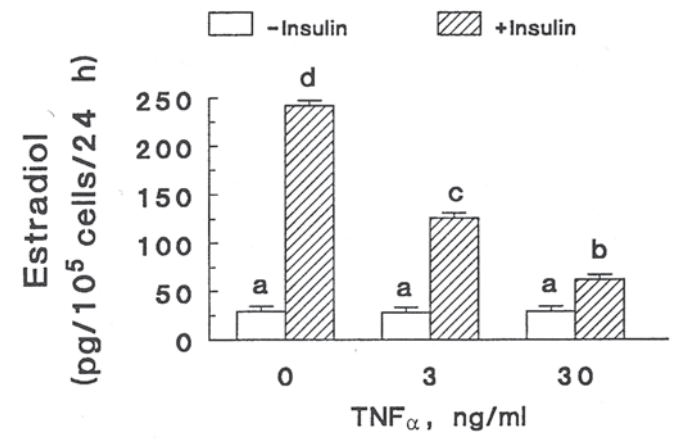

Fig. 1. Effect of TNF-α on basal and insulin-induced estradiol production by granulosa cells from small (1–5 mm) follicles (Exp. 1). Granulosa cells were cultured for 2 d in the presence of 10% FCS as described in Materials and Methods and then treated in serum-free medium with 500 ng/mL of testosterone with or without 1 μg/mL of insulin and the various doses of TNF-α for an additional 24 h. Values are means from four separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common superscript differ ($p < 0.05$).

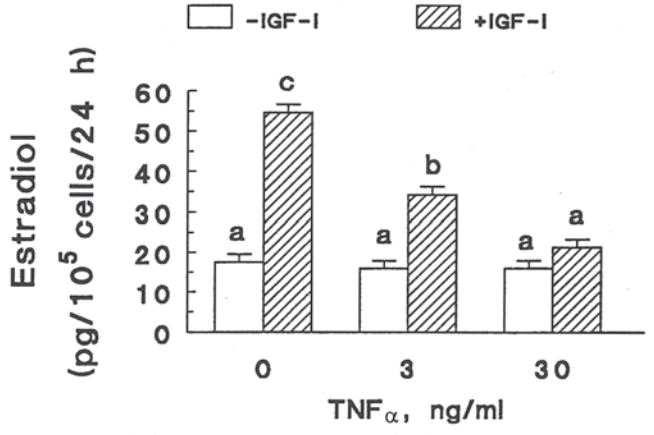

Fig. 2. Effect of TNF-α on basal and IGF-I-induced estradiol production by granulosa cells from small (1–5 mm) follicles (Exp. 2). Granulosa cells were cultured as described for Fig. 1 except that 100 ng/mL of IGF-I was used instead of insulin. Values are means from three separate experiments. Means without a common superscript differ ($p < 0.05$).

Table 1
Effect of 1-d Treatment of TNF-α on Granulosa Cell Numbers

Dose of TNF-α ng/mL	Cells/well, $\times 10^{-5}$			
	Exp. 1[a]		Exp. 2[b]	
	–Insulin	+Insulin	–IGF-I	+IGF-I
0.0	0.55	1.27[c]	0.54	1.62
3.0	0.53	1.16[c]	0.54	1.68
30.0	0.58	1.02[d]	0.53	1.51
SEM	0.05	0.05	0.06	0.06

[a]Values are means from four separate experiments.
[b]Values are means from three separate experiments.
[c,d]Within a column, means without a common superscript differ ($p < 0.05$).

Effect of TNF-α on IGF-I-Induced Granulosa Cell Estradiol Production

The objective of these experiments was to evaluate the effect of TNF-α on IGF-I-induced estradiol production by granulosa cells of small follicles (Exp. 2). In the absence of TNF-α, IGF-I increased granulosa cell estradiol production by threefold (Fig. 2). In the absence of IGF-I, basal estradiol production by granulosa cells from small follicles was not affected ($p > 0.10$) by TNF-α (Fig. 2). However, in the presence of IGF-I, 3 and 30 ng/mL of TNF-α significantly reduced granulosa cell estradiol production by 37 and 61%, respectively (Fig. 2). Basal and IGF-I-induced cell numbers were not affected ($p > 0.10$) by TNF-α (Table 1).

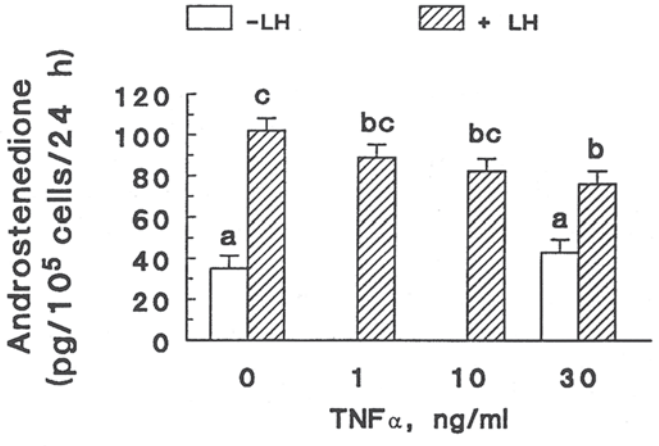

Fig. 3. Effect of TNF-α on basal and LH-stimulated androstenedione production by thecal cells from large (≥8 mm) follicles (Exp. 3). Thecal cells were cultured for 2 d in the presence of 10% fetal calf serum, and then treated in serum-free media with either 0 (open panels) or 100 ng/mL (hatched panels) of LH for an additional 2 d. During the last 2 d of culture, TNF-α (0, 1, 10, or 30 ng/mL) was also added to the medium. Values are means of three separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means without a common superscript differ ($p < 0.05$).

Effect of TNF-α on Basal and Luteinizing Hormone (LH)-Induced Thecal Cell Androstenedione Production

The objective of these experiments was to evaluate the effect of TNF-α on basal and LH-induced androstenedione production by thecal cells of large follicles (Exp. 3). In the absence of TNF-α, LH increased ($p < 0.01$) androstenedione production by 2.9-fold (Fig. 3). Dosage of TNF-α tended ($p < 0.08$) to inhibit LH-induced androstenedione production by thecal cells, such that LH-induced increase in androstenedione production was 1.8-fold above controls

Table 2
Effect of 2-d Treatment of TNF-α on Thecal Cell Numbers

Dose of TNF-α ng/mL	Dose of LH, ng/mL	Cells/well, $\times 10^{-5}$ Exp. 3[a]	Exp. 4[b]
0	0	1.45	1.78[c]
0	100	1.30	1.63[c]
1	100	1.38	1.57[c]
10	100	1.39	1.88[d]
30	0	1.29	1.66[c]
30	100	1.38	1.97[d]
SEM		0.05	0.06

[a]Values are means from three separate experiments.
[b]Values are means from three separate experiments; cells were also cultured in the presence of 1 μg/mL of insulin.
[c,d]Within a column, means without a common superscript differ ($p < 0.05$).

in the presence of 30 ng/mL of TNF-α (Fig. 3). Basal androstenedione production was not affected ($p > 0.10$) by TNF-α (Fig. 3). Cell numbers were not affected ($p > 0.10$) by LH or TNF-α (Table 2).

Effect of TNF-α on Insulin- and LH Plus Insulin-Induced Thecal Cell Androstenedione Production

The objective of these experiments was to evaluate the effect of TNF-α on insulin- and LH plus insulin-induced androstenedione production by thecal cells of large follicles (Exp. 4). In the absence of TNF-α, but presence of insulin, LH increased ($p < 0.001$) androstenedione production by 34-fold (Fig. 4). Dosage of TNF-α inhibited ($p < 0.05$) the LH-induced androstenedione production by thecal cells, such that LH-induced increase in androstenedione production was reduced 48% to 15-fold above controls in the presence of 30 ng/mL of TNF-α (Fig. 4). In these same experiments, 10 and 30 ng/mL of TNF-α increased ($p < 0.05$) cell numbers by 15 and 21%, respectively, in the presence of LH (Table 2).

Presence of Specific TNF-α Binding Sites on Granulosa and Thecal Cells

The objective of these experiments was to evaluate whether specific binding of ^{125}I-TNF-α existed on granulosa and thecal cells. Granulosa cells from small follicles and thecal cells from large follicles were cultured for 3 d in the presence of 10% FCS, and then cells were washed and ^{125}I-TNF-α binding assays were conducted directly in the culture wells. For the first series of experiments (Exp. 5), approx 150,000 dpm of ^{125}I-TNF-α were incubated for 0–4 h at 25°C. Both granulosa and thecal cells specifically bound ^{125}I-TNF-α in a time-dependent manner with maximal binding achieved between 2 and 4 h of incubation at 25°C (Fig. 5). For the second series of experiments (Exp. 6), binding of 50,000–300,000 dpm of ^{125}I-TNF-α to granulosa and thecal cells revealed that TNF-α binding was of

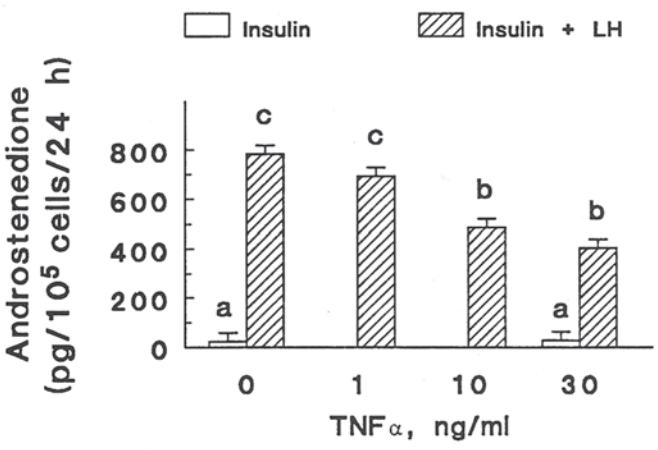

Fig. 4. Effect of TNF-α on insulin- and LH-stimulated androstenedione production by thecal cells from large (≥8 mm) follicles (Exp. 4). Thecal cells were cultured for 2 d in the presence of 10% fetal calf serum, and then treated in serum-free media with 1 μg/mL of insulin and either 0 (open panels) or 100 ng/mL (hatched panels) of LH for an additional 2 d. During the last 2 d of culture, TNF-α (0, 1, 10, or 30 ng/mL) was also added to the medium. Values are means of three separate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Means with a common superscript differ ($p < 0.05$).

high affinity with an estimated dissociation constant (K_d) of $24 \pm 6 \times 10^{-12}$ *M* and $16 \pm 2 \times 10^{-12}$ *M* for granulosa and thecal cells, respectively, and of low capacity with an estimated receptor concentration of $0.148 \pm .003$ fmol/10^6 cells and $0.172 \pm .041$ fmol/10^6 cells for granulosa and thecal cells, respectively. A representative Scatchard plot *(21)* for each cell type is shown in Fig. 6.

Discussion

An inhibitory effect of recombinant bovine TNF-α (74% inhibition with 30 ng/mL of TNF-α in the present study) on insulin-induced estradiol production by granulosa cells of small follicles in the bovine has been supported by studies of Spicer and Alpizar *(13)*, who found that addition of 100 ng/mL of recombinant human TNF-α brought about a 72% reduction in FSH plus insulin-induced estradiol production by granulosa cells from small follicles; granulosa cells from large follicles in the previous report showed no significant response to TNF-α on estradiol production. It is not surprising that recombinant human and bovine TNF-α yield similar results, since the amino acid sequence of bovine TNF-α shares a 81% homology with that of human *(22,23)*. We also found that in the absence of FSH, TNF-α completely blocked the IGF-I-induced estradiol production by granulosa cells in the present study. Previous studies have focused on the effect of TNF-α on FSH-induced granulosa cells steroidogenesis. Others *(24–26)* found that recombinant human TNF-α inhibits (by 70%) FSH-induced estradiol production by rat undifferentiated

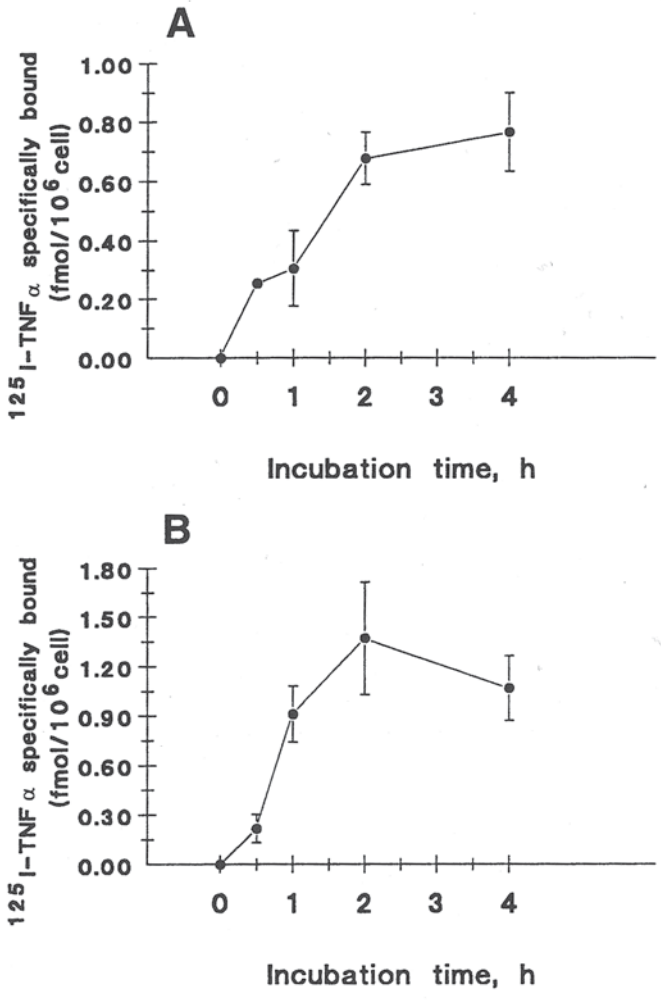

Fig. 5. Time-course of specific ^{125}I-TNF-α binding in granulosa **(A)** and thecal **(B)** cells (Exp. 5). Granulosa and thecal cells were cultured for 3 d in the presence of 10% fetal calf serum, and then cells were washed and TNF-α binding assays were conducted as described in Materials and Methods. Values are means of three separate experiments; within each replicate experiment, each time-point was derived from duplicate total binding wells and duplicate nonspecific binding wells.

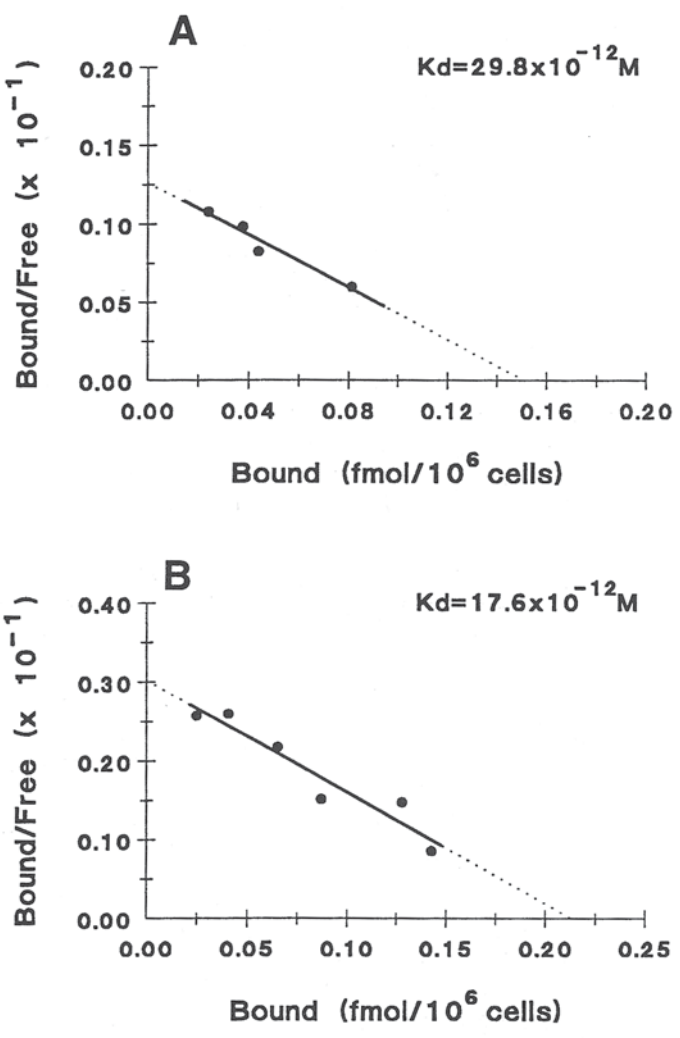

Fig. 6. Estimation of the number of TNF-α receptor sites and dissociation constant in bovine granulosa **(A)** and thecal **(B)** cells (Exp. 6). Granulosa and thecal cells were cultured as described in Fig. 5. Cells were incubated with increasing amounts of ^{125}I-TNF-α in the presence or absence of excess unlabeled TNF-α (*see* Materials and Methods) and subject to Scatchard analysis *(21)*. Data presented are from one representative experiment for each cell type.

granulosa cells. TNF-α also inhibits FSH-induced estrogen production by human granulosa cells *(27–29)*. In addition, others have observed that human TNF-α inhibited gonadotropin-dependent progesterone production by rat granulosa cells *(26,30–32)*, porcine granulosa cells *(33)*, and human granulosa cells *(29,34)*. Although the mechanism by which TNF-α inhibits insulin- and IGF-I-induced aromatase in bovine granulosa cells will require further elucidation, the inhibitory effect of TNF-α on gonadotropin-induced steroidogenesis of murine and porcine granulosa cells is multifaceted, since TNF-α is capable of inhibiting:

1. FSH-induced cAMP accumulation.
2. FSH-induced LH receptor formation.
3. Post-cAMP-mediated events.
4. Cholesterol side-chain cleavage activity and mRNA.
5. 3β-hydrosysteroid reductase activity *(30,32,33)*.

TNF-α had no effect on bovine and rat granulosa cell viability *(13,25,35)*. Collectively, these results indicate that TNF-α is a potent inhibitor of steroidogenesis and aromatase activity of undifferentiated granulosa cells from cattle and rats. The demonstration that TNF-α and IL-6 are more effective inhibitors of aromatase activity in small vs large follicles *(13,27,36)* adds support to the hypothesis that increased numbers of immune cells within large follicles *(19)* and regressing corpora lutea *(15,17,18)* during the follicular phase of the cycle may inhibit differentiation of smaller follicles, thereby preventing premature differentiation of future ovulatory follicles. During various diseases, when serum TNF-α levels are elevated *(6,7)*, TNF-α may act to inhibit ovarian follicular development.

We observed that TNF-α reduced LH-induced thecal cell androstenedione production by approx 50%. Previously, TNF-α was found to inhibit LH-induced androgen production by purified rat thecal cells *(37–39)*, but not androgen production by porcine thecal cells *(40)* in vitro. In addition, recombinant human TNF-α inhibited (by 40%) insulin plus LH-induced progesterone production by bovine thecal cells in vitro *(35)*. The mechanism by which TNF-α inhibits steroidogenesis of bovine thecal cells is unclear, but based on studies with rat thecal cells may involve several loci including:

1. A decrease in number of LH/hCG receptors.
2. A decrease in LH/hCG-induced cAMP accumulation.
3. A decrease in post-cAMP-mediated events.
4. A decrease in protein kinase activity.
5. A decrease in 17α-hydroxylase/17:20-lyase activity *(37,38)*.

As with granulosa cells, TNF-α has no effect on murine thecal cell viability *(37)*. Thus, it appears that TNF-α affects steroidogenesis of both granulosa and thecal cells of cattle and rats. This is in contrast to the effects of IL-2, where IL-2 inhibits steroidogenesis of bovine granulosa cells and not thecal cells *(41)*. Because thecal cell androgen production is needed for maximum estradiol production by preovulatory follicles of cattle *(42)*, an inhibitory effect of TNF-α on thecal cell steroidogenesis would prevent maximum estradiol production from occurring.

As previously reported for bovine granulosa cells *(13)*, TNF-α had no effect on numbers of granulosa cells from small follicles or thecal cells from large follicles grown in basal serum-free medium or medium containing IGF-I or LH. Similarly, others observed that TNF-α had no effect on bovine *(35)* or murine *(37)* thecal cell numbers in the absence or presence of LH/hCG. In contrast, Fukuoka et al. *(43)* reported that recombinant human TNF-α increased (by fivefold) basal and insulin-stimulated ^{3}H-thymidine incorporation of porcine granulosa cells obtained from small follicles (1–2 mm) and grown in medium containing 10% FCS. Similarly, others *(44,45)* reported that 10 ng/mL of TNF-α increased proliferation (by nearly twofold) of human granulosa cells collected from >16 mm follicles and cultured in the presence of insulin and/or 10% serum. TNF-α also increased ^{3}H-thymidine incorporation by human ovarian carcinoma cells *(46)*. Darbon et al. *(30)* reported that 50 ng/mL of TNF-α reduced granulosa cell numbers by 18% when cultured in serum-free media. In the present study, we observed that recombinant bovine TNF-α (30 ng/mL) inhibited insulin-induced numbers of granulosa cells from small follicles by 20% and potentiated insulin-induced numbers of thecal cells from large follicles by 21%. Perhaps the effect of TNF-α on insulin-induced cell proliferation, whether stimulatory or inhibitory, is dependent on the type of cell, the in vitro environment (e.g., presence of other hormones or growth factors), or both. Nonetheless, under serum-free conditions, the effect of TNF-α on granulosa and thecal cell proliferation is minor compared with its effect on steroidogenesis.

Results of the present study also suggest that ovarian granulosa and thecal cells may have high-affinity receptors for TNF-α. The TNF-α receptor has a single extracellular domain that binds TNF-α with high affinity *(47)*. Although TNF-α receptors have not been identified previously in granulosa or thecal cells of cattle, specific binding has been reported in rat ovarian cells *(48)*, porcine granulosa cells *(33)*, and porcine corpora lutea *(49)*. These results suggest that the effect of TNF-α on ovarian cells is likely mediated through binding to its receptor.

In summary, the inhibitory effect of TNF-α on ovarian follicular steroidogenesis in cattle encompasses both granulosa and thecal cells as well as LH, insulin, and IGF-I action. The physiologic relevance of such a broad ovarian effect of TNF-α is unclear. Because systemic concentrations of TNF-α in bovine plasma during infection *(6,7)* are within the range of effective doses observed in the present study, TNF-α may play an endocrine role and inhibit ovarian function during infection and disease. The source of the increase in follicular fluid TNF-α during follicular growth is uncertain *(14)*, but may derive, in part, from macrophages that infiltrate ovarian follicles and corpora lutea *(15–19)*. Levels of TNF-α in bovine and human follicular fluid *(14,50–52)* are also within the range of effective doses observed in the present and previous studies. Thus, TNF-α may act as an intraovarian regulator of follicular function during the normal estrous cycle. Collectively, the present and previous results indicate that TNF-α may play a role in regulating follicular function during normal follicular growth as well as during disease states where systemic TNF-α may be elevated.

Materials and Methods

Reagents and Hormones

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, trypsin, pronase E, collagenase, hyaluronidase, DNase, insulin (bovine, 25.7 U/mg), and fetal calf serum (FCS) obtained from Sigma Chemical Co. (St. Louis, MO); bovine LH (USDA-bLH-B5, LH activity 2.1 x NIH-LH-S1 U/mg; FSH activity < 1.0% by weight) obtained from the National Hormone and Pituitary Program (Baltimore, MD); testosterone obtained from Steraloids (Wilton, NH); recombinant bovine TNF-α obtained from CIBA-GEIGY (Basle, Switzerland); and recombinant human IGF-I obtained from R&D Systems (Minneapolis, MN) were used.

Cell Culture

Ovaries of beef and dairy cattle obtained at slaughter from a nearby abattoir were brought to the laboratory on ice (within 120 min) and processed as previously described for obtaining granulosa cells *(13,53)* and thecal cells *(54,55)*.

Briefly, granulosa cells were collected separately from the small (≤5 mm) follicles, based on surface diameter of follicles, by aspirating the follicular fluid. The granulosa cells were separated from follicular fluid by centrifugation (200*g* for 7.5 min) and by washing twice in serum-free medium (1:1 mixture of DMEM and Ham's F-12 containing 0.12 m*M* gentamicin and 38.5 m*M* sodium bicarbonate). Thecal cells, on the other hand, were obtained from walls of large (≥8 mm) follicles by hand separation of theca layer and subsequent exposure to enzymatic digestion as described previously *(54,55)*. Thecal and granulosa cell suspensions containing $1–3 \times 10^5$ viable cells, as ascertained by trypan blue exclusion method *(56)*, were seeded in each well (Falcon multiwell plates-#3047; Becton Dickinson and Co., Lincoln Park, NJ) containing 1 mL of medium. The percentage of red blood cells (RBC) at the time of plating was 35 ± 3% for granulosa cells from small follicles, and 23 ± 3% for thecal cells from large follicles. Cultures of both granulosa and thecal cells were incubated at 38.5°C in a 5% CO_2 atmosphere, and the medium was changed every 22–26 h as described by Langhout et al. *(53)*. To obtain optimal attachment, cells were maintained in the presence of 10% FCS for the first 2 d of culture. After this time, cells were washed twice with 0.5 mL serum-free medium and incubations continued for 1 or 2 d in serum-free medium with or without added hormones, unless stated otherwise. For studies evaluating the effects of TNF-α on granulosa cells, cells were treated with 500 ng/mL of testosterone with or without 1 μg/mL of insulin or 100 ng/mL of IGF-I and various doses of TNF-α for 1 d (i.e., from d 2 to 3 of culture). For studies evaluating the effects of TNF-α on thecal androstenedione production, thecal cells were treated with or without 100 ng/mL of LH and/or 1 μg/mL of insulin with various doses of TNF-α for 2 d (i.e., from d 2 to 4 of culture).

Cell Enumeration

At the termination of each experiment, the granulosa and thecal cells attached to the bottom of wells were scraped free, after treatment with 0.5 mL of trypsin (0.25% in 0.15 *M* NaCl) for 20 min at 25°C, and counted with a Coulter counter (Model Zm, Coulter Electronics Hialeah, FL) as previously described *(53,55)*.

Assessment of 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 *(13)*. Estradiol production increases linearly from 4 to 24 h of incubation under these conditions *(13)*.

Hormone Radioimmunoassay (RIA)

Estradiol RIA

Concentrations of estradiol in culture medium were determined by double-antibody RIA as previously described *(13,57)*. The intra- and interassay coefficients of variation were 10.6 and 17.6% for the estradiol RIA.

Androstenedione RIA

Concentrations of androstenedione in culture medium were determined by solid-phase RIA using the Immuchem Covalent Coat Diagnostic Kit provided by ICN Biomedicals, Inc. (Costa Mesa, CA) as previously described *(41,55)*. The intra- and interassay coefficients of variation were 12.0 and 12.4%, respectively.

TNF-α Iodination and Radioreceptor Assay

TNF-α was iodinated using a chloramine-T method as previously described *(6)*. ^{125}I-TNF-α and free ^{125}I was separated on a 30-cm Sephadex G50 column. The ^{125}I-TNF-α binding assays were conducted directly in the culture wells in an assay volume of 400 μL (0.25% BSA in PBS, pH = 7.5). Incubation temperature was 25°C for various times. Specific binding was determined as the difference between total binding and nonspecific binding; nonspecific binding was determined using 0.5 μg/well of excess unlabeled recombinant bovine TNF-α.

Statistical Analysis

Analyses of granulosa cells from small follicles and thecal cells from large follicles were based on the results of three or more experiments each conducted on a separate pool of cells obtained from 20 to 38 ovaries (average 30 ± 4 ovaries) for granulosa cells, and 6 to 10 follicles from 4 to 8 cows for thecal cells. The main effects and their interactions were assessed by general linear models procedure of SAS *(58)* and expressed as least-squares means and standard errors. Specific differences in cell growth and steroid production (expressed as pg/10^5 cells/24 h by dividing the total steroid produced per well by the number of cells determined by Coulter counting) among treatments were tested using Fisher's protected least-significant difference procedure suggested by Ott *(59)*. Significance was declared at ($p < 0.05$) unless noted otherwise.

Acknowledgments

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