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Interleukin-2 affects steroidogenesis and numbers of bovine ovarian granulosa cells but not thecal cells *in vitro*[1]

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The effect of recombinant bovine interleukin-2 (IL-2) on steroidogenesis and numbers of bovine ovarian granulosa and thecal cells has been studied. Granulosa cells have been examined from both small (surface diameter ⩽ 5 mm) and large (⩾ 8 mm) follicles, whereas thecal cells from only large follicles were utilized. Estradiol and progesterone production per cell by granulosa cells from large follicles was 2- to 3-times greater than those from small follicles. Increasing doses of IL-2 significantly attenuated FSH-induced estradiol production by cells from small follicles but not large follicles. In general, progesterone production per cell by granulosa cells was almost double that of thecal cells. Moreover, IL-2 significantly attenuated FSH-induced progesterone production by granulosa cells from small and large follicles but had no effect on LH-induced progesterone or androstenedione production by thecal cells. Co-treatment of TNFα with IL-2 enhanced the responsiveness of granulosa cells to IL-2. The effect of IL-2 on the numbers of granulosa and thecal cells were studied independently under serum-free conditions and media enriched with 10% fetal calf serum. In serum-free medium containing insulin, IL-2 dosage significantly increased numbers of granulosa cells from large follicles, whereas IL-2 had no effect on numbers of granulosa cells from small follicles or thecal cells from large follicles. When cells were grown in medium enriched with serum, increasing doses of IL-2 significantly inhibited numbers without affecting viability of granulosa cells from small follicles, but had no effect on numbers of thecal cells. Thus, it appears that granulosa cells are more sensitive to IL-2 than are thecal cells.

Keywords: interleukin-2; ovary; granulosa cells; thecal cells; steroidogenesis

Introduction

Interleuken-2 (IL-2) was one of the first hormones recognized in the immune system as having a role in T-cell growth and in the function of other cell types including B-cells, monocytes, and lymphokine-activated killer cells (for reviews see: Robb, 1984; Harrison & Campbell, 1988; Minami *et al.*, 1993; Janssen *et al.*, 1994). In addition to its effects on immune cells, IL-2 and another T-cell cytokine, IL-6, regulates proliferation of endocrine cells including anterior pituitary cells (Gorospe & Spangelo, 1993; Arzt *et al.*, 1993). In spite of the fact that IL-6 and other cytokines such as tumor necrosis factor-α (TNFα) seem to affect ovarian cell function (Adashi, 1990; Brannstrom & Norman, 1993; Gorospe & Spangelo, 1993; Spicer *et al.*, 1995), the role of IL-2 in ovarian cell proliferation and steroidogenesis is practically unexplored. The meagre data available indicates that IL-2 stimulates cell proliferation and inhibits hCG-induced progesterone production by luteinized human granulosa cells (Wang *et al.*, 1991). In swine, IL-2-like activity is present in ovarian follicular fluid and levels decrease as follicles enlarge (Takakura *et al.*, 1989). Although the intraovarian source of IL-2 has not been identified, it is likely that some of the IL-2 in follicular fluid is derived from lymphocytes including T-cells that infiltrate ovarian follicles and corpora lutea (Standaert *et al.*, 1991; Brannstrom *et al.*, 1993a; 1994). Also, detection of soluble IL-2 receptors in serum and ascites in patients with epithelial ovarian cancer (Barton *et al.*, 1993; Hurteau *et al.*, 1994) further suggests a possible role of IL-2 in ovarian function. Hence, the objectives of our studies were to evaluate the effect of IL-2 on steroidogenesis and proliferation of granulosa and thecal cells.

Results

Effect of IL-2 on basal and FSH-induced granulosa cell estradiol production

The objective of these experiments was to evaluate and compare the effect of IL-2 on estradiol production by granulosa cells of small and large follicles. In the absence of FSH, basal estradiol production, expressed as picogram of estradiol per 10^5 cells, by small-follicle granulosa cells was not affected ($P > .05$) by IL-2 (Table 1). In the presence of FSH, there was a significant inhibitory effect of IL-2 dosage on estradiol production by granulosa cells from small follicles, whereas IL-2 had no significant effect on estradiol production by cells from large follicles (Figure 1). At 30 ng/ml of IL-2, estradiol production was 53% of control values in cultures of small-follicle granulosa cells. Estradiol production by large-follicle granulosa cells was two-fold greater than that by small-follicle granulosa cells (Figure 1).

Effect of IL-2 on granulosa cell and thecal progesterone production

The objective of these experiments was to evaluate and compare the effect of IL-2 on progesterone production by granulosa cells of small and large follicles and by thecal cells of large follicles. In the absence of FSH, basal progesterone production, expressed as nanogram of progesterone per 10^5 cells, by granulosa cells from small follicles was not affected ($P > .05$) by IL-2 (Table 1). However, in the presence of FSH, the dosage of IL-2 significantly reduced granulosa cell progesterone production by 24% and 39% in cells from small and large follicles, respectively (Figure 2). In contrast, IL-2 had no effect on LH-induced progesterone production by thecal cells (Table 2).

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Table I Lack of effect of one-day treatment of IL-2 on cell numbers and basal progesterone and estradiol production by cells from small follicles.[a]

Dose of IL-2, ng/ml	*Cells/well ($\times 10^{-5}$)*	*Progesterone (ng/10^5 cells/24 h)*	*Estradiol (pg/10^5 cells/24 h)*
0	4.30	25.6	245
10	4.19	22.7	263
30	3.99	25.5	348
Pooled SE	.16	1.4	59

[a]Values are means from three separate experiments

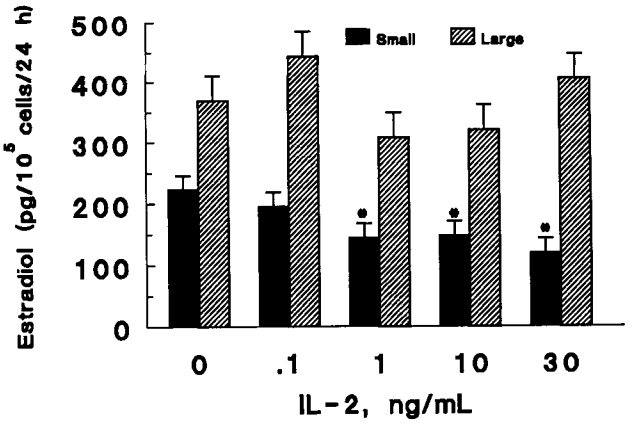

Figure 1 Effect of IL-2 on FSH-induced estradiol production (pg/10^5 cells/24 h) by granulosa cells from small ■ and large ▨ follicles. Granulosa cells were cultured for 2 days in the presence of 10% FCS as described in Materials and methods and then treated in serum-free medium with 50 ng/ml of FSH, 1 μg/ml of insulin, and 1 μg/ml of testosterone with or without the various doses of IL-2 for an additional 24 h. Values are means from four separate experiments for small follicles and three separate experiments for large follicles. Estradiol production in the absence of FSH but in the presence of insulin averaged 76±23 and 229±53 pg/10^5 cells/24 h for cells from small and large follicles, respectively. *Within follicle size, mean differs ($P<.05$) from controls without IL-2

The timing of the inhibitory effect of IL-2 on FSH-induced progesterone production by granulosa cells from large follicles was evaluated and presented in Figure 3. The inhibitory effect of 30 ng/ml of IL-2 was first observed at 24 h but not 4 or 8 h.

Effect of IL-2 on thecal cell androstenedione production

The objective of these experiments was to evaluate the effect of IL-2 on androstenedione production by thecal cells of large follicles. Dosage of IL-2 had no significant effect on LH-induced androstenedione production by thecal cells, expressed as picogram of androstenedione per 10^5 cells (Table 2).

Interaction between IL-2 and TNFα on granulosa cell progesterone production

The objective of this experiment was to determine the interaction between low doses of IL-2 and TNFα on progesterone production by granulosa cells of large follicles since previous studies have reported interactions between interleukins and TNFα (Dinarello, 1989; Minami *et al.*, 1993). The interaction between IL-2 and TNFα on progesterone production by granulosa cells from large follicles is depicted in Figure 4. As expected, 1 ng/ml of IL-2 or TNFα alone had no effect on progesterone production. However, in the presence of 1 ng/ml of TNFα, 1 ng/ml of IL-2 significantly inhibited progesterone production induced by FSH (Figure 4). Progesterone production in the presence of 10 ng/ml of TNFα was lower than controls regardless if IL-2 was included in the medium (Figure 4).

Table II Lack of effect of one-day treatment of IL-2 on cell numbers and progesterone and androstenedione production by thecal cells from large follicles cultured in serum-free medium containing LH[a]

Dose of IL-2, ng/ml	*Cells/well ($\times 10^{-5}$)*	*Progesterone (ng/10^5 cells/24 h)*	*Androstenedione (pg/10^5 cells/24 h)*
0.0	1.59	28	145
0.1	1.67	28	141
1.0	1.66	29	147
10.0	1.77	29	131
30.0	1.75	23	142
0.0[b]	1.59	18*	22*
Pooled SE	.05	3	8

[a]Values are means from three separate experiments. [b]Cells treated with insulin but not LH. *Within a column, mean differs ($P<.05$) from those treated with LH

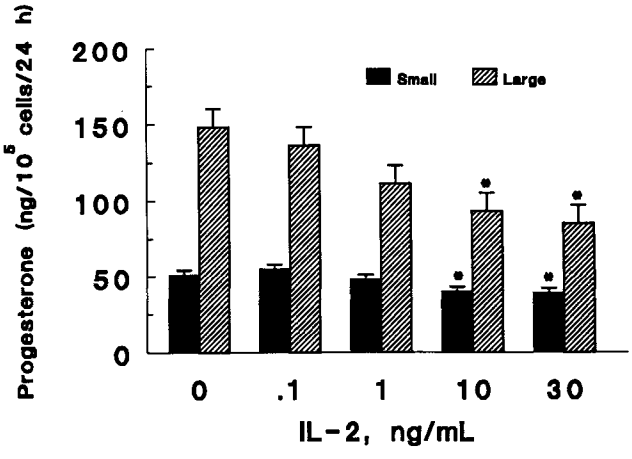

Figure 2 Effect of IL-2 on FSH-induced progesterone production (ng/10^5 cells/24 h) by granulosa cells from small ■ and large ▨ follicles. Cells were cultured as described for Figure 1. Progesterone production in the absence of FSH but in the presence of insulin averaged 40±3 and 106±12 ng/10^5 cells/24 h for cells from small and large follicles, respectively. Values are means from four separate experiments for small follicles and three separate experiments for large follicles. *Within follicle size, mean differs ($P<.05$) from controls without IL-2

Effect of IL-2 on granulosa and thecal cell numbers in serum-free medium

The effects of IL-2 on the numbers of granulosa cells from small and large follicles (Figure 5) as well as thecal cells collected from large follicles (Table 2) were compared under serum-free culture conditions in the presence of gonadotropins and insulin. Dose of IL-2 (10 and 30 ng/ml) significantly increased numbers of granulosa cells from large follicles (Figure 5), whereas IL-2 had no significant effect on numbers of granulosa cells from small follicles (Figure 5) or thecal cells from large follicles (Table 2). In the absence of FSH, IL-2 also had no effect ($P>.05$) on numbers of granulosa cells from small follicles in serum-free medium (Table 1).

Effect of IL-2 on granulosa and thecal cell numbers in medium enriched with serum

The effects of IL-2, day of culture, and experiment on numbers of thecal and granulosa cells in medium enriched

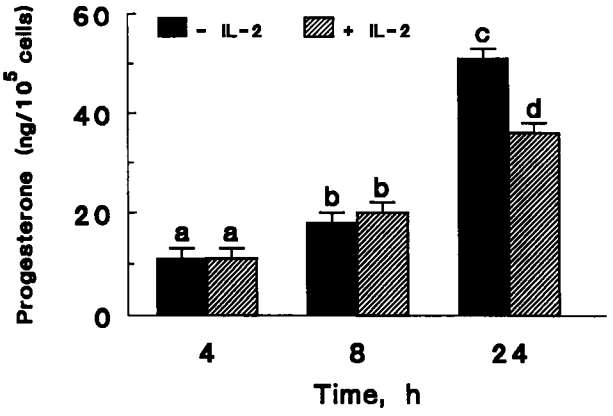

Figure 3 Effect of incubation time on the inhibitory effect of IL-2 on FSH-induced progesterone production by granulosa cells from large follicles. Cells were cultured for 2 days in presence of 10% FCS as described in Materials and methods and then treated in serum-free medium with 50 ng/ml of FSH and 1 μg/ml of insulin with ▨ or without ■ 30 ng/ml of IL-2 for an additional 4, 8 or 24 h. [a,b,c,d]Means without a common superscript differ ($P<.05$)

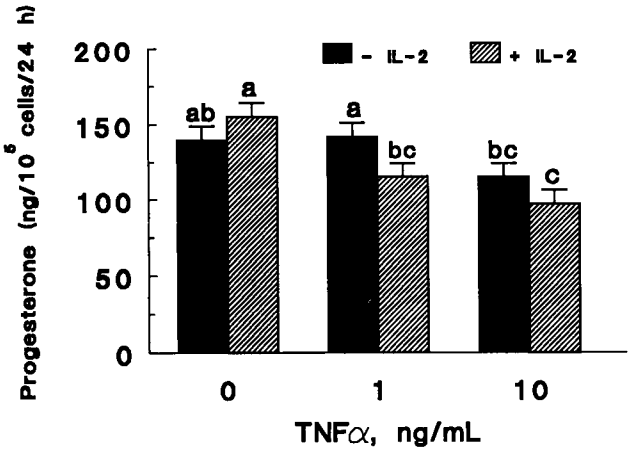

Figure 4 Interaction between IL-2 and TNFα on progesterone production by granulosa cell from large follicles. Cells were cultured for 2 days in the presence of 10% FCS as described in Materials and methods and then treated in serum-free medium with 50 ng/ml of FSH and 1 μg/ml of insulin with ▨ or without ■ IL-2 (1 ng/ml) and TNFα (0, 1 or 10 ng/ml) for an additional 2 days. Values are means from four separate experiments. *Means without a common letter differ ($P<.05$)

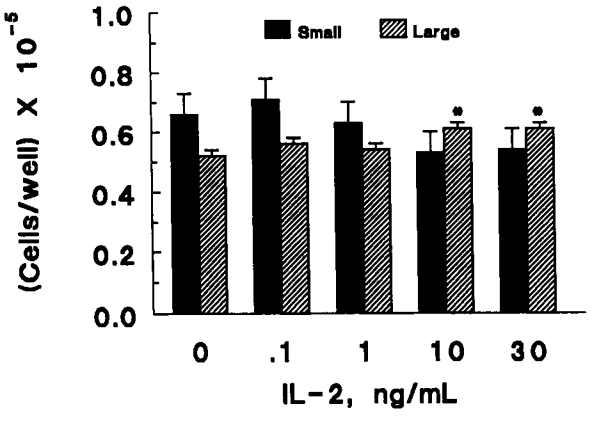

Figure 5 Effect of IL-2 on numbers of granulosa cells from small ■ and large ▨ follicles. Cells were cultured as described for Figure 1. Cell numbers in the absence of FSH but in the presence of insulin averaged 0.52±.06 and 0.53±03 × 10^5 cells/well for cells from small ($n=4$ experiments) and large ($n=3$ experiments) follicles, respectively. *Within follicle size, mean differs ($P<.05$) from controls without IL-2

with 10% FCS were determined (Figure 6). A twofold growth of both granulosa and thecal cells occurred in control cultures between day 1 and day 2 of treatment in this enriched medium. Increased doses of IL-2 significantly suppressed numbers of granulosa cells but not thecal cells (Figure 6). Cell growth differed ($P<.05$) between experiments and increased ($P<.05$) from the first to second day of treatment. The interaction between day of treatment and dose of IL-2 on granulosa and thecal cell growth was not significant. In a second series of experiments conducted in an identical manner as those in Figure 6, viability of granulosa cells from small follicles was evaluated. After 2 days of treatment, 30 ng/ml of IL-2 had no effect on viability of granulosa cells; viability averaged 95.5 and 94.5 ± 5% in control and IL-2-treated cultures, respectively ($n=3$ experiments).

Discussion

An inhibitory effect of recombinant bovine IL-2 (47% inhibition with 30 ng/ml of IL-2 in the present study) on FSH-induced estradiol production by granulosa cells of small follicles in the bovine has been supported by studies of Spicer & Alpizar (1994), who found that addition of 100 ng/ml of recombinant human IL-2 brought about a 45% reduction in FSH-induced estradiol production by granulosa cells from small follicles; granulosa cells from large follicles in both reports showed no significant response to IL-2 on FSH-induced estradiol production. Thus, in spite of the fact that

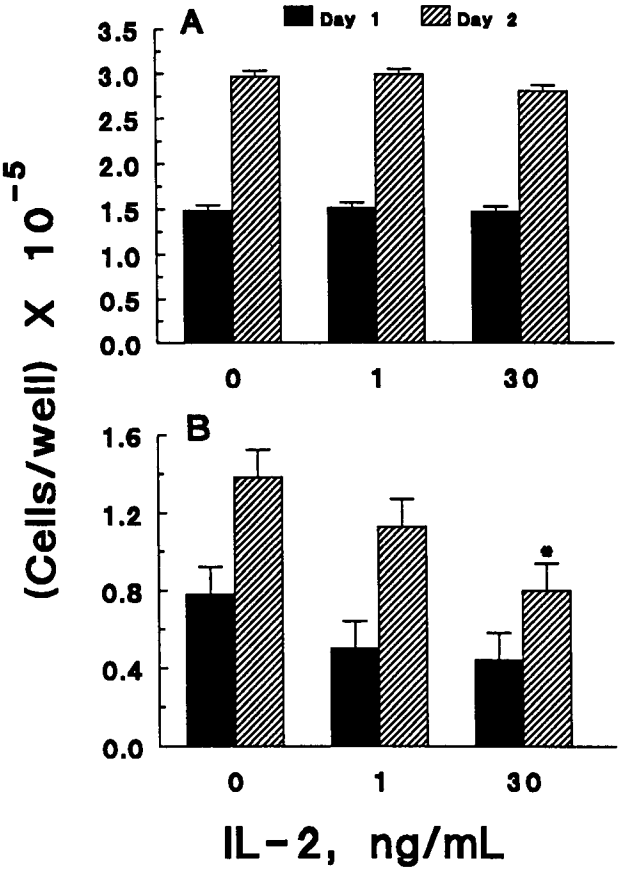

Figure 6 Effect of IL-2 on numbers of thecal cells from large follicles (**a**) and granulosa cells from small follicles (**b**) on two consecutive days of treatment in medium containing 10% FCS (■, Day 1 of treatment; ▨, Day 2 of treatment). Granulosa cells were cultured for 4 days in the presence of 10% FCS as described in Materials and methods. During the last 2 days, the various doses of IL-2 were added to medium containing 10% FCS. Values are means from seven separate experiments for thecal cells and three separate experiments for granulosa cells. *Within day of treatment, mean differs ($P<.05$) from controls without IL-2

the amino acid sequence of bovine IL-2 shares a 65% homology with that of human IL-2 (Cerretti *et al.*, 1986), similar effects were observed. In comparison, Adashi *et al.* (1989) and Kasson & Gorospe (1989) found that recombinant human IL-2 had no effect on FSH-induced estradiol production by rat undifferentiated granulosa cells. Similarly, Fukuoka *et al.* (1992a) observed that human IL-2 had no significant effect on hCG-stimulated estradiol production by luteinized human granulosa cells. Collectively, these results indicate that IL-2 does not affect aromatase activity of highly differentiated granulosa cells from cattle and humans. Why IL-2 inhibits estradiol production by undifferentiated granulosa cells of cattle and not rats remains to be determined, but may be due, in part, to the fact that rat IL-2 and human IL-2 share only a 50% amino acid homology (Cerretti *et al.*, 1986). In support of this suggestion, recombinant rat IL-2 induced an increase in plasma ACTH concentrations in rats whereas recombinant human IL-2 had no effect (Naito *et al.*, 1989). In addition, recombinant human TNFα is much less effective in competing for ^{125}I-mTNFα binding sites on murine cells than is recombinant mouse TNFα (Smith *et al.*, 1986). Similar to these differential effects of IL-2 on cells from small and large follicles, we have previously shown that granulosa cells from large bovine follicles are 40-times less sensitive to another T-cell cytokine, IL-6, than cells from small bovine follicles (Alpizar & Spicer, 1994). The demonstration that IL-2 and IL-6 are more effective inhibitors of aromatase activity in small versus large follicles adds support to the hypothesis that increased numbers of immune cells within large follicles (Brannstrom *et al.*, 1993a) and regressing corpora lutea (Standaert *et al.*, 1991) 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 IL-2 levels are elevated (Komorowski, 1992; Nyabenda *et al.*, 1993), IL-2 may act to inhibit ovarian follicular development.

An inhibitory effect of IL-2 on FSH-induced progesterone production by bovine granulosa cells in a dose-dependent manner is reported here for the first time. In addition, we found that low doses of TNFα enhanced the inhibitory effect of low doses of IL-2 on progesterone production. Although a synergism between IL-2 and other cytokines on steroidogenesis has not been reported, a previous study has indicated that other cytokines (e.g. interferon-α) enhance the mitogenic effect of IL-2 on endothelial cells (Cozzolino *et al.*, 1993). In addition, 1 ng/ml of TNFα enhances the inhibitory effect of 1 ng/ml of IL-1 on progesterone production by human luteinized granulosa cells (Fukuoka *et al.*, 1992b). Previously, a 2-day treatment of recombinant human IL-2 inhibited hCG-stimulated progesterone production by luteinized human granulosa cells (Wang *et al.*, 1991), whereas Fukuoka *et al.* (1992a) found no effect of a 6-day treatment of recombinant human IL-2 on hCG-stimulated progesterone production by luteinized human granulosa cells. In comparison, recombinant human IL-2 has been shown to either stimulate after 2 days of treatment (Kasson & Gorospe, 1989) or have no effect after 3 days of treatment (Gottschall *et al.*, 1988) on FSH-induced progesterone production by rat granulosa cells, and have no effect on basal progesterone production by rabbit luteal cells (Chudaska & Schlegel, 1993). In cultured intact preovulatory rat follicles, recombinant human IL-2 had no effect on basal or LH-stimulated progesterone accumulation (Brannstrom *et al.*, 1993b). Additional studies are required to clarify whether differences in culture conditions or species differences in IL-2 structure account for these variable effects of human IL-2 on rat granulosa cell progesterone production, since, as mentioned, rat IL-2 and human IL-2 share only a 50% amino acid homology (Cerretti *et al.*, 1986) and rat but not human IL-2 induces release of ACTH in rats (Naito *et al.*, 1989). Another T-cell cytokine, IL-6, also inhibits progesterone production by rat granulosa cells (Gorospe *et al.*, 1992). Why IL-2 would inhibit progesterone production and not estradiol production by granulosa cells of large bovine follicles is uncertain but may be one mechanism whereby premature luteinization of granulosa cells of preovulatory follicles is prevented while allowing for an increased capacity to produce estradiol. Whether differences in the number of IL-2 receptors between small and large follicles account for the differential response to IL-2 between small and large follicles remains to be elucidated.

We observed that IL-2 had no significant effect on LH-induced androstenedione or progesterone production by thecal cells, and to our knowledge this is a new finding. Previous studies have not evaluated the effect of IL-2 on thecal androgen production, but IL-1 has been shown to inhibit hCG-induced androgen production by rat whole ovarian homogenates (Hurwitz *et al.*, 1991) and by rat (Moore & Moger, 1991) and porcine (Mauduit *et al.*, 1992) Leydig cells *in vitro*. In cultured rat Leydig cells, recombinant mouse IL-2 inhibits hCG-induced testosterone production (Guo *et al.*, 1990). However, recombinant bovine IL-1 has no effect on LH-induced progesterone production by bovine luteal cells (Nothnick & Pate, 1990). In cultured intact preovulatory rat follicles, recombinant human IL-2 had no effect on androstenedione accumulation (Brannstrom *et al.*, 1993b), whereas recombinant human TNFα increased androstenedione accumulation (Roby & Terranova, 1988). In addition, recombinant human TNFα inhibited LH-induced progesterone production by bovine thecal cells *in vitro* (Brunswig-Spickenheier & Mukhopadhyay, 1993), and cAMP-induced testosterone production by mouse Leydig cells *in vitro* (Li *et al.*, 1995). Previously, we have reported that TNFα inhibits estradiol production by bovine granulosa cells (Spicer & Alpizar, 1994) and androstenedione production by bovine thecal cells (Spicer *et al.*, 1995). Thus, it appears that the steroidogenic effect of IL-2 on bovine ovarian follicles is specific to the membrana granulosa, whereas TNFα affects steroidogenesis of both granulosa and thecal cells of cattle. Similarly in rats, the T-cell cytokine, IL-6, inhibits steroidogenesis of granulosa cells (Gorospe *et al.*, 1992) but not thecal cells (Hurwitz *et al.*, 1991), whereas TNFα inhibits steroidogenesis of both granulosa (Emoto & Baird, 1988; Adashi *et al.*, 1989) and thecal cells (Andreani *et al.*, 1991; Zachow *et al.*, 1993). Whether thecal cell steroidogenesis in species other than the bovine is affected by IL-2 that is homologous to that species remains to be determined. The physiological relevance of the finding that IL-2 affects granulosa cell steroidogenesis and not thecal cell steroidogenesis of large bovine follicles is unclear, but is consistent with our previously stated hypothesis that IL-2 may allow for maximal estradiol production at the same time it inhibits luteinization (i.e., progesterone production) of granulosa cells. Because thecal cell androgen production is needed for maximum estradiol production by preovulatory follicles of cattle (Spicer & Echternkamp, 1986), the lack of an effect of IL-2 on thecal cell steroidogenesis would allow for this maximum estradiol production to occur. Further research is needed to determine if the lack of thecal cell-response to IL-2 is due to a lack of IL-2 receptors.

As previously reported for human granulosa cells (Wang *et al.*, 1991), IL-2 increased numbers of granulosa cells from large follicles grown in serum-free medium. However, IL-2 had no effect on numbers of granulosa cells from small follicles or thecal cells from large follicles grown in serum-free medium. Similarly, Fukuoka *et al.* (1992c) reported that recombinant human IL-2 had no effect on ^{3}H-thymidine incorporation of porcine granulosa cells obtained from small follicles (1–2 mm) and cultured under serum-free conditions. Spicer & Alpizar (1994) observed that recombinant human IL-2 (100 ng/ml) inhibited numbers of granulosa cells from small follicles by 27% but had no effect on numbers of granulosa cells from large follicles. Consistent with these findings, Fukuoka *et al.* (1989) found that granulosa cells from large porcine follicles (6–11 mm) were less responsive

to recombinant human IL-1 than those from small (1–2 mm) follicles grown in 10% calf serum. When cells were grown in medium enriched with 10% calf serum in the present study where cell growth is more vigorous than in serum-free medium (Langhout *et al.*, 1991), IL-2 at 30 ng/ml inhibited numbers of granulosa cells from small follicles, whereas numbers of thecal cells were not affected. Thus, similar to its effect on steroidogenesis, IL-2 has specific effects on granulosa cells but not on thecal cells in cattle.

In summary, the inhibitory effect of IL-2 on ovarian follicular steroidogenesis in cattle is confined to granulosa cells. The physiologic relevance of such a specific ovarian effect of IL-2 is unclear, since the levels of IL-2 in bovine follicular fluid and serum are unknown. However, concentrations of IL-2 in human plasma (Komorowski, 1992; Licinio *et al.*, 1993) and serum (Shimomura *et al.*, 1991; Nyabenda *et al.*, 1993) are within the range of effective doses observed in the present study. Furthermore, IL-2-like activity is present in porcine ovarian follicular fluid and levels decrease as follicles enlarge (Takakura *et al.*, 1989). The source of intraovarian IL-2 is unknown, but may derive, in part, from lymphocytes including T-cells that infiltrate ovarian follicles and corpora lutea (Standaert *et al.*, 1991: Brannstrom *et al.*, 1993a; 1994). Thus, IL-2, like IL-6, may act as intraovarian regulator of follicular function. Collectively, the present and previous results indicate that IL-2 may play a role in regulating follicular function during normal follicular growth as well as during disease states where systemic IL-2 may be elevated.

Materials and methods

Reagents and hormones

Dulbecco's Modified Eagles 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); FSH (ovine, F1913, FSH activity 15 × NIH-FSH-S1 U/mg) from Scripps Laboratories (San Diego, CA); bovine LH (USDA-bLH-B5, LH activity 2.1 × NIH-LH-S1 U/mg; FSH activity < 1.0% by weight) obtained from the National Hormone and Pituitary Program (Baltimaore, MD); testosterone obtained from Steraloids (Wilton, NH); recombinant bovine TNFα obtained from CIBA-GEIGY (Basle, Switzerland); and recombinant bovine IL-2 (5×10^8 U/mg and 1–2 EU endotoxin per mg) obtained from American Cyanamid Co. (Princeton, NJ) were used.

Cell culture

Ovaries of beef and dairy cattle obtained at slaughter from a nearby abbatoir were brought to the laboratory on ice (within 120 min) and processed as described by Langhout *et al.*, (1991) and Alpizar & Spicer (1994) for obtaining granulosa cells, and Roberts & Skinner (1990) for obtaining thecal cells. Briefly, granulosa cells were collected separately from the small (⩽ 5 mm) and large (⩾ 8 mm) follicles, based on surface diameter of follicles, by aspirating the follicular fluid. Thecal cells, on the other hand, were obtained from walls of large follicles by hand separation of theca layer and subsequent exposure to enzymatic digestion as described previously (Roberts & Skinner, 1990). 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 mM gentamicin and 38.5 mM sodium bicarbonate). Finally, the cell suspension containing 1 to 3×10^5 viable cells, as ascertained by trypan blue exclusion method (Adashi *et al.*, 1987), was seeded in each well (Falcon multiwell plates - No. 3047; Becton Dickinson & Co., Lincoln Park, NJ) containing 1 ml of medium. The percentage of red blood cells (RBC) at the time of plating was 26.0 ± 5.8% and 26.4 ± 1.9%, respectively, for those from small and large follicles. The RBC contamination for thecal cells was 29.9 ± 3.6%. 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 to 26 h as described by Langhout *et al.* (1991). To obtain optimal attachment, cells were maintained in the presence of 10% FCS for the first 2 days of culture. After this time, cells were washed twice with 0.5 ml serum-free medium and incubations continued for 1 or 2 days in serum-free medium with or without added hormones, unless stated otherwise. For studies evaluating the effects of IL-2 on granulosa cells, cells were treated with 50 ng/ml of FSH, 1 μg/ml of insulin and 1 μg/ml of testosterone with or without .1, 1.0, 10 and 30 ng/ml of IL-2 for 1 day (i.e., from days 2 to 3 of culture). For studies evaluating the effects of IL-2 on thecal androstenedione and progesterone production, thecal cells were treated with 100 ng/ml of LH and 1 μg/ml of insulin with or without 0.1, 1.0, 10, or 30 ng/ml of IL-2 for 1 day (i.e., from days 2 to 3 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 described by Langhout *et al.* (1991) and Barano & Hammond (1985).

Assessment of functional aromatase activity

Functional aromatase activity was assessed during a 24 h exposure of granulosa cells to 1 μg/ml of testosterone as previously described (Spicer & Alpizar, 1994). Estradiol production increases linearly from 4 to 24 h of incubation under these conditions (Spicer & Alpizar, 1994).

Hormone radioimmunoassay (RIA)

Estradiol and progesterone RIA The production of estradiol and(or) progesterone by the granulosa and thecal cells was assessed by RIA as described by Spicer and Enright (1991) and expressed as picogram or nanogram per cell production by dividing the total hormone produced per well by the number of cells determined by Coulter counting. The intra- and inter-assay coefficients of variation were 14.2 and 17.5% for estradiol, and 17.3 and 18.9% for progesterone, respectively.

Androstenedione RIA The androstenedione was quantified by solid-phase RIA using the Immuchem Covalent Coat Diagnostic Kit provided by ICN Biomedicals, Inc. (Costa Mesa, CA). In the assay, the antibody was covalently bound to the inner surface of a polypropylene tube. Bound and free hormone were separated by vacuum aspiration. Increasing volumes of unextracted conditioned medium (10–150 μl) resulted in parallel displacement of specific binding of ^{125}I-androstenedione in the RIA. The assay sensitivity, defined as 90% of total binding, was 4.4 pg/tube. The intra- and inter-assay coefficients of variation were 9.9 and 11.2%, respectively.

Statistical analysis

Analyses of granulosa cells from small and large follicles and thecal cells were based on the results of three or more experiments conducted on separate pools of ovaries numbering 20 to 38 (average 30 ± 4 ovaries). The main effects and their interactions were assessed by GLM procedures of SAS (1988) and expressed as least squares means and standard errors. Specific differences in cell growth and steroid produc-

tion among treatments were tested using Fisher's protected least significant difference procedure suggested by Ott (1977). Significance was declared at ($P < .05$) unless noted otherwise.

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