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Effects of Estradiol on Bovine Thecal Cell Function In Vitro: Dependence on Insulin and Gonadotropins*

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ABSTRACT

The objective of this study was to evaluate the influence of estradiol (E2) on proliferation and steroid production by thecal cells obtained from large (≥8 mm) follicles of cattle. Five experiments evaluated the effect of various doses of E2 during a 2-d exposure in serum-free medium on hormone-induced steroidogenesis and cell proliferation. In LH-treated thecal cells of experiment 1, 300 ng/mL of E2 decreased progesterone production by 30% and increased androstenedione production to 5.8-fold of controls. In the absence of LH, both 3 and 300 ng/mL of E2 increased progesterone production. In experiment 2, in the presence of insulin and LH, 3, 30, and 300 ng/mL of E2 decreased progesterone production (by 17 to 36%), whereas 3 ng/mL of E2 decreased and 300 ng/mL of E2 increased androstenedione production. Doses of LH (3 to 30 ng/mL) tested in experiment 3 increased (to as much as 3.7-fold) progesterone production by thecal cells and E2 attenuated this stimulatory effect by 40%. In contrast, E2 amplified the stimulatory effect of LH on androstenedione production in experiment 3. In experiment 4, E2 (300 ng/mL) decreased IGF-I- and insulin-induced thecal cell progesterone production by 70 to 77%, whereas E2 increased basal, IGF-I, and insulin-induced androstenedione production. In experiment 5, in the presence of insulin, 10 to 1000 ng/mL of E2 had no effect on [^{125}I]-IGF-I binding to thecal cells, whereas 10 and 100 ng/mL of E2 increased and 1000 ng/mL of E2 decreased progesterone production by thecal cells. Estradiol had no consistent effect on thecal cell numbers among the 5 experiments. These results support the hypothesis that E2 may act as a paracrine factor to directly regulate hormone-induced steroid production by thecal cells without affecting cell numbers or numbers of insulin-like growth factor type I receptors.

(**Key words:** estradiol, thecal cell, insulin-like growth factor, steroidogenesis)

Abbreviation key: E2 = estradiol, **ER** = estrogen receptor, **FCS** = fetal calf serum, **hCG** = human chorionic gonadotropin.

INTRODUCTION

During preovulatory follicular growth, increases in systemic concentrations of estradiol (**E2**) in cattle influence a variety of functions including stimulation of estrous behavior, ovulatory gonadotropin surges, and gamete transport (Spicer and Echternkamp, 1986; Buhi, 2002; Orikasa and Sakuma, 2003). In lactating dairy cows, plasma and follicular fluid estradiol concentrations are negatively correlated with daily milk yield (Beam and Butler, 1998) and are reduced by high environmental temperatures (Wolfenson et al., 1997; Wilson et al., 1998). Because these factors have been associated with reduced reproductive efficiency, an association between reduced concentrations of estradiol and decreased reproductive efficiency is plausible. Direct effects of estradiol on ovarian cell function were first reported in cattle in 1979 (Fortune and Hansel, 1979a,b), and shown to be important modulators of FSH action in granulosa cells of rats (Richards et al., 1976; Tonetta et al., 1985) and pigs (Veldhuis et al., 1982; 1984). However, the effect of estradiol on thecal cell function has not been well described. Estrogen receptors (**ER**) and their mRNA (α and β) have been detected in bovine (Rosenfeld et al., 1999; Bao et al., 2000; Berisha et al., 2002; Van Den Broeck et al., 2002), porcine (Slomczynska and Wozniak, 2001), and human (Brandenberger et al., 1998; Sharma et al., 1999; Jakimiuk et al., 2002) granulosa and thecal cells. Furthermore, E2 directly alters ovarian granulosa cell steroidogenesis in several species, and ERα knockout female mice are unable to ovulate in response to exogenous gonadotropins (Couse et al., 1999). Specifically, Fortune and Hansel (1979a) and Langhout et al. (1991) found that E2 inhibited progesterone production by bovine granulosa cells in the presence of fetal calf serum (**FCS**) and insulin, respectively. Hunter and Armstrong (1987) and Gilling-Smith et al. (1997) found that E2 inhibited basal

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and LH-induced progesterone production by porcine and human thecal cells, respectively. Voge et al. (2004) found that E2 decreased IGF binding protein-2, -3, and -4 mRNA levels in bovine thecal cells. However, the effect of E2 on hormone-induced steroidogenesis of ovarian thecal cells in cattle has not been thoroughly evaluated. Therefore, the objective of the present study was to examine the effects of estradiol on basal and hormone-induced steroidogenesis of thecal cells from bovine ovarian follicles.

MATERIALS AND METHODS

Reagents and Hormones

Reagents were Dulbecco's modified Eagle medium, Ham's F12 medium, insulin (bovine; 28 U/mg), pronase E, collagenase, hyaluronidase, DNase, FCS, and E2 obtained from Sigma Chemical Co. (St. Louis, MO); bovine LH (L1913; LH activity 2× NIH-LH-S1 U/mg) obtained from Scripps Laboratories (San Diego, CA); and recombinant human IGF-I obtained from R&D Systems (Minneapolis, MN).

Cell Culture

Ovaries were obtained at a nearby commercial abattoir from beef and dairy cattle after slaughter. The ovaries were transported to the laboratory (<120 min) and processed as described previously (Langhout et al., 1991; Stewart et al., 1995; Spicer et al., 2001a). Thecal cells from large (8 to 22 mm) follicles were isolated via enzyme digestion as previously described (Stewart et al., 1995; Spicer and Stewart, 1996a; Spicer and Chamberlain, 1998). The purity of thecal cells prepared this way has been estimated at >95% (Roberts and Skinner, 1990a,b; Spicer and Stewart, 1996a). Isolated cells were resuspended in medium containing 1.25 mg/mL of collagenase and 0.5 mg/mL of DNase to prevent clumping of the cells. Basal medium consisted of a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 containing 0.12 m*M* gentamicin, 2.0 m*M* glutamine, and 38.5 m*M* sodium bicarbonate. Viable cells were then determined by the trypan blue exclusion method; viability averaged 92 ± 1% for thecal cells. Approximately 2×10^5 viable cells were placed in each well of Falcon 24-well plates (no. 3047, Becton Dickinson, Lincoln Park, NJ) containing 1 mL of medium. Cells were cultured at 38.5°C in a 95% air/5% CO_2 atmosphere, and the medium was changed every 24 h. Thecal cells grown in this culture system establish monolayers and retain hormonally responsive androstenedione and progesterone production (Stewart et al., 1995; Spicer and Stewart, 1996a; Spicer and Chamberlain, 1998).

Experiment 1 was conducted to determine the effect of 0, 3, or 300 ng/mL of E2 on thecal cell steroidogenesis in the absence of growth factors. Thecal cells from large (≥8 mm) follicles were cultured for 2 d in medium containing 10% FCS and then cultured in serum-free medium for an additional 2 d with LH (0 or 30 ng/mL) and E2 (0, 3, or 300 ng/mL). Medium was changed every 24 h. The doses of E2 were selected to represent typical average values within the wide range of concentrations of E2 in follicular fluid of dairy (Stewart et al., 1996; Spicer et al., 2001b) and beef (Spicer et al., 1986a,b, 1987) cows.

Experiment 2 was conducted to evaluate the effect of 0, 30, or 300 ng/mL of E2 on thecal cell steroidogenesis in the presence of LH and insulin. Thecal cells were cultured as in experiment 1 except that, during the last 2 d of culture, E2 (0, 30, or 300 ng/mL), LH (30 ng/mL), and insulin (100 ng/mL) were added to the medium. The doses of E2 were selected as per experiment 1, and doses of LH and insulin were selected based on previous studies (Stewart et al., 1995; Spicer and Stewart, 1996a,b; Spicer and Chamberlain, 1998).

Experiment 3 was conducted to evaluate the effect of E2 on thecal cell steroid production in the presence of insulin and either low or high doses of LH. Thecal cells from large (≥8 mm) follicles were cultured for 2 d in medium containing 10% FCS and then cultured in serum-free medium for an additional 2 d with insulin (1000 ng/mL), LH (0, 3, or 30 ng/mL), and E2 (0 or 300 ng/mL). Medium was changed every 24 h. The doses of E2, LH, and insulin were selected based on experiments 1 and 2.

Experiment 4 was designed to compare the effect of E2 on basal, IGF-I, and insulin-induced thecal cell steroidogenesis. Thecal cells from large (≥8 mm) follicles were cultured for 2 d in medium containing 10% FCS and then cultured in serum-free medium for an additional 2 d with LH (30 ng/mL) and IGF-I (0 or 30 ng/mL) or insulin (0 or 100 ng/mL). During the last 2 d of culture, E2 (0 or 300 ng/mL) was added to the medium. Medium was changed every 24 h. Doses of E2, LH, insulin, and IGF-I were selected based on experiments 1 and 2.

Experiment 5 was conducted to evaluate the effect of 0, 10, 100, or 1000 ng/mL of E2 on thecal IGF-I receptors and steroidogenesis. Thecal cells from large follicles (≥8 mm) were cultured for 2 d in medium containing 10% FCS and then cultured in serum-free medium for an additional 2 d with insulin (10 ng/mL) and E2 (0, 10, 100, or 1000 ng/mL). Medium was changed every 24 h. The doses of E2 and insulin were selected based on experiments 1 and 2. Because E2 was dissolved in ethanol, all treatments without E2 received the same vol-

ume of ethanol (final ethanol content = 0.003%) as a vehicle control for all experiments.

Determination of Cell Number

Medium was collected from individual wells and frozen at –20°C for subsequent hormone analyses. Cells were gently washed twice with 0.9% saline (500 μL), exposed to 500 μL of trypsin solution (0.25% wt/vol) for 20 min at 25°C, and then scraped from each well. Cell aggregates were disrupted by pipetting the cell suspension back and forth through a 500-μL pipette tip 3 to 5 times, and cells were then diluted in 9 mL of 0.9% saline, and counted using a Coulter counter (model Zm; Coulter Electronics, Hialeah, FL) as previously described (Langhout et al., 1991; Stewart et al., 1995).

Assays

Concentrations of progesterone and androstenedione in culture medium were determined by radioimmunoassays as previously described (Langhout et al., 1991; Stewart et al., 1995; Spicer et al., 1996). The intra- and interassay coefficients of variation were 10 and 16% for progesterone radioimmunoassay and 7 and 13% for the androstenedione assay. Because E2 cross-reacted in the androstenedione assay, unspent medium samples were assayed and these values were subtracted from E2-treated samples values.

Numbers of binding sites for [^{125}I]-IGF-I in bovine thecal cells were determined as previously described (Stewart et al., 1995; Spicer and Stewart, 1996b; Spicer, 2001). Briefly, numbers of specific [^{125}I]-IGF-I binding sites were determined by incubating cells with a saturating amount (approximately 250,000 cpm) of [^{125}I]-IGF-I for 16 h at 4°C in the 17-mm wells of the multiwell culture plate. Nonspecific binding was measured in the presence of 500 ng of unlabeled IGF-I. After the 16-h incubation, wells were washed, and the cells were solubilized with 1 *N* NaOH and counted in a gamma counter (counter efficiency = 75%).

Statistical Analyses

Each experiment contained 3 replicates per treatment and each experiment was replicated 3 to 4 times with different pools of thecal cells. Each pool of thecal cells was obtained from 4 to 8 follicles collected from 3 to 6 animals. Data are presented as the least squares means (± SE) of measurements from 9 to 12 culture wells. Main effects (i.e., hormone, dose, experimental replicate) and interactions were assessed using the GLM procedure of SAS (SAS Institute, 1999). Steroid production was expressed as nanograms or picograms per 10^5 cells per 24 h, and cell numbers at the termination of the experiment were used for this calculation. Specific differences in cell numbers, steroid production, and [^{125}I]-IGF-I binding among treatments were determined using Fisher's protected least significant difference procedure (Ott, 1977).

RESULTS

Experiment 1

Progesterone production. Luteinizing hormone alone increased ($P < 0.001$) progesterone production (to 3-fold of controls) by thecal cells, and 300 ng/mL of E2 (but not 3 ng/mL) inhibited ($P < 0.05$) this increase by 30% (Figure 1A). In the absence of LH, E2 increased ($P < 0.05$) basal progesterone production to 1.4- to 1.7-fold of controls (Figure 1A).

Androstenedione production. Luteinizing hormone alone increased ($P < 0.01$) androstenedione production (to 2.5-fold of controls) by thecal cells, and 3 and 300 ng/mL of E2 amplified ($P < 0.05$) this increase (Figure 1B). In the absence of LH, 300 ng/mL of E2 (but not 3 ng/mL) increased ($P < 0.05$) basal androstenedione production to 11-fold of controls; in the presence of LH, this increase was to 5.8-fold of controls (Figure 1B).

Cell numbers. In contrast to steroid production, neither dose of E2 nor LH had an effect ($P > 0.10$) on cell numbers, which averaged $1.01 \pm 0.05 \times 10^5$ cell per well (data not shown).

Experiment 2

Progesterone production. Both E2 ($P < 0.05$) and insulin ($P < 0.001$) affected progesterone production (Figure 2A). In the presence of insulin plus LH, 3, 30, and 300 ng/mL of E2 decreased ($P < 0.05$) progesterone production by 17 to 36% (Figure 2A); in the absence of insulin but in the presence of LH, 300 ng/mL of E2 had no effect ($P > 0.10$) on progesterone production. In the absence of E2, 100 ng/mL of insulin increased ($P < 0.05$) progesterone production to 3.5-fold of controls (Figure 2A).

Androstenedione production. In the presence of insulin plus LH, 3 ng/mL of E2 decreased ($P < 0.05$), 30 ng/mL of E2 had no effect ($P > 0.10$), and 300 ng/mL of E2 increased ($P < 0.05$) androstenedione production (Figure 2B). In the absence of insulin but presence of LH, 300 ng/mL of E2 increased ($P < 0.05$) androstenedione production to 3.8-fold of controls (Figure 2B). In the absence of E2, 100 ng/mL of insulin amplified ($P < 0.05$) LH-induced androstenedione production to 5.7-fold of controls (Figure 2B).

Cell numbers. Doses of 3 and 30 ng/mL of E2 had no effect ($P > 0.10$) on cell numbers, whereas 300 ng/

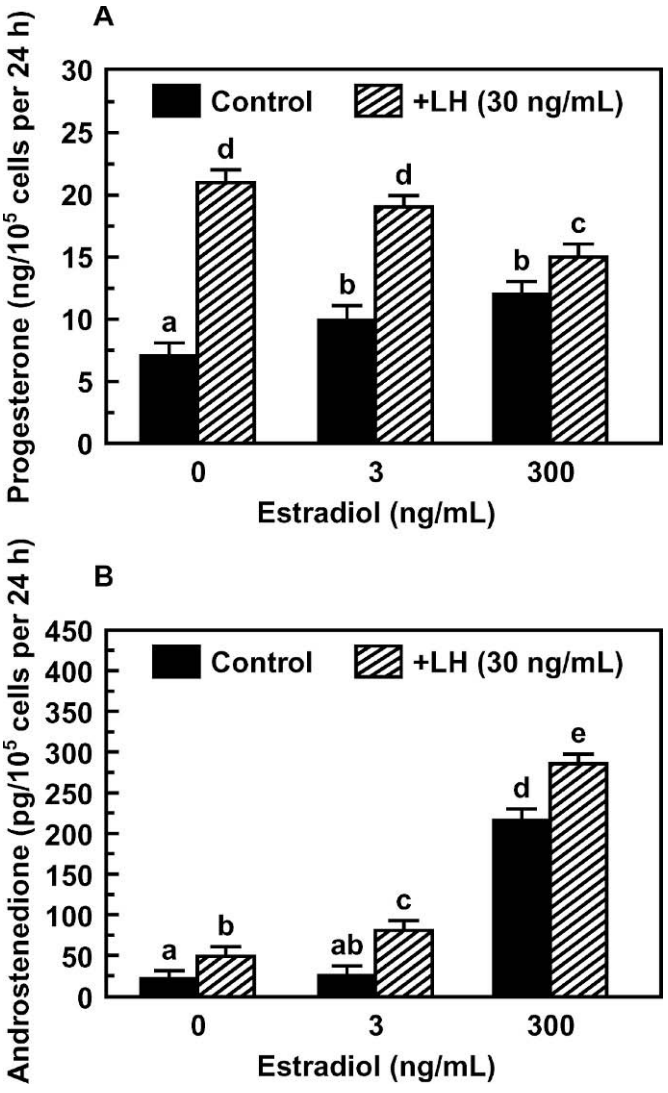

Figure 1. Effect of estradiol (E2) on basal and LH-induced progesterone (panel A) and androstenedione (panel B) production by thecal cells from large follicles (experiment 1). Thecal cells were cultured for 4 d as described in Materials and Methods. During the last 2 d of culture, cells were treated in serum-free medium with 0 or 30 ng/mL of LH with or without various doses of E2. Values are means from 4 separate experiments. [a,b,c,d,e]Within a panel, means without a common letter differ ($P < 0.05$).

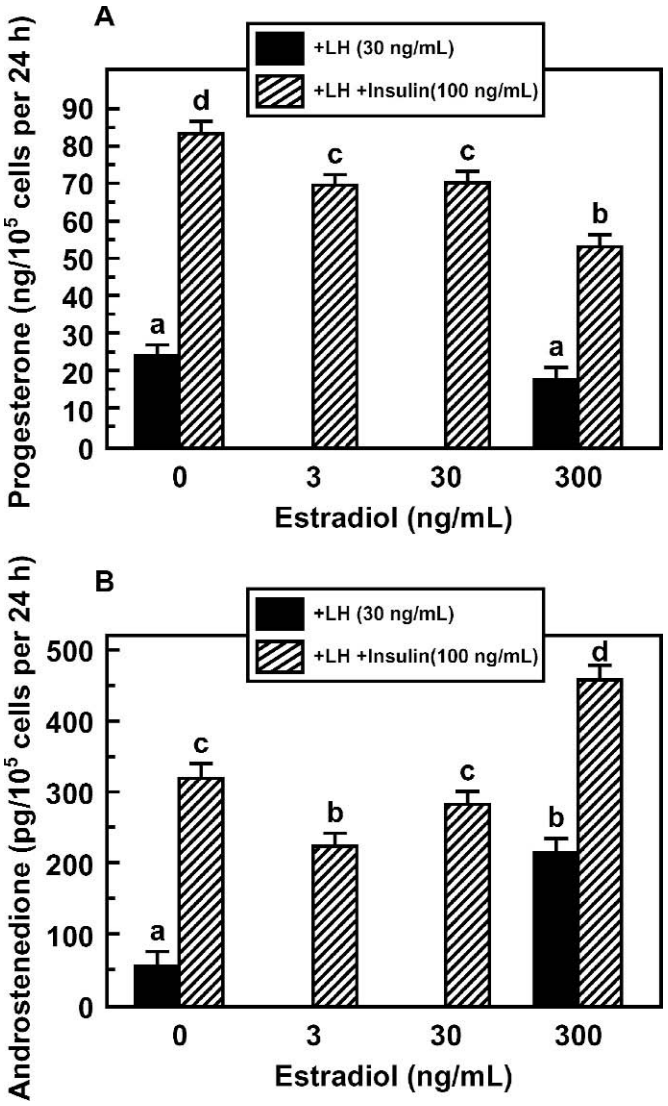

Figure 2. Effect of estradiol (E2) on insulin plus LH-induced progesterone (panel A) and androstenedione (panel B) production by thecal cells from large follicles (experiment 2). Thecal cells were cultured for 4 d as described in Materials and Methods. During the last 2 d of culture, cells were treated in serum-free medium with 100 ng/mL of insulin and 30 ng/mL of LH with or without various doses of E2. Values are means from 3 separate experiments. [a,b,c,d]Within a panel, means without a common letter differ ($P < 0.05$).

mL of E2 weakly increased (i.e., 9% increase) cell numbers in the presence but not absence of 100 ng/mL of insulin (Table 1).

Experiment 3

Progesterone production. In the presence of insulin (1000 ng/mL), 3 and 30 ng/mL of LH increased ($P < 0.05$) progesterone production to 3.0- and 3.7-fold of controls, respectively (Figure 3A). However, E2 attenuated ($P < 0.05$) this LH-induced progesterone production by thecal cells. Specifically, E2 reduced progesterone production by 32 and 26% ($P < 0.05$) in the presence of 3 and 30 ng/mL of LH, respectively (Figure 3A).

Androstenedione production. In the presence of insulin (1000 ng/mL), 3 and 30 ng/mL of LH increased ($P < 0.05$) androstenedione production to 5.9- and 9.2-fold of controls, respectively (Figure 3B). Estradiol dramatically increased ($P < 0.05$) basal and LH-induced androstenedione production (Figure 3B).

Table 1. Effect of 2-d treatment of estradiol (E2) in combination with LH, insulin, and IGF-I on numbers of thecal cells from large (≥8 mm) bovine follicles.

Experiment	Dose (ng/mL) E2	LH	Insulin	IGF-I	Cell number ($\times 10^5$ per well)
2	0	30	0	0	2.28[a]
	0	30	100	0	3.71[b]
	3	30	100	0	3.84[bc]
	30	30	100	0	3.92[bc]
	300	30	0	0	2.24[a]
	300	30	100	0	4.04[c]
	SEM				0.11
4	0	30	0	0	0.51[a]
	300	30	0	0	0.61[ab]
	0	30	0	30	0.94[c]
	300	30	0	30	0.85[c]
	0	30	100	0	0.90[c]
	300	30	100	0	0.80[bc]
	SEM				0.08
5	0	0	10	0	1.13[ab]
	10	0	10	0	1.23[b]
	100	0	10	0	1.24[b]
	1000	0	10	0	1.01[a]
	SEM				0.04

[a,b,c]Within an experiment, means without a common letter differ ($P < 0.05$).

Cell numbers. Neither LH nor E2 affected ($P > 0.10$) cell numbers, which averaged $2.80 \pm 0.06 \times 10^5$ cells per well (data not shown).

Experiment 4

Progesterone production. In the presence of LH, 300 ng/mL of E2 decreased ($P < 0.05$) progesterone (Figure 4A) production by 72%. Similarly, E2 decreased ($P < 0.05$) progesterone production in the presence of IGF-I (by 70%) and insulin (by 77%) (Figure 4A).

Androstenedione production. In the presence of LH, 300 ng/mL of E2, 30 ng/mL of IGF-I, and 100 ng/mL of insulin each increased ($P < 0.05$) androstenedione production to 6.8-, 8.2-, and 6.4-fold of controls, respectively (Figure 4B). Estradiol amplified ($P < 0.05$) the IGF-I- and insulin-induced increase in androstenedione production by 21 and 66%, respectively, but this response was less than additive (Figure 4B).

Cell numbers. Both IGF-I and insulin (but not LH) increased ($P < 0.05$) cell numbers (Table 1). However, 300 ng/mL of E2 had no effect ($P > 0.10$) on IGF-I- or insulin-induced cell numbers (Table 1).

Experiment 5

Progesterone production. Dose of E2 ($P < 0.01$) had a biphasic influence on progesterone production by thecal cells (Figure 5A). In the presence of low (10 ng/mL) insulin but absence of LH, 10 and 100 ng/mL of E2 increased ($P < 0.05$) progesterone production to 1.3- and 1.5-fold, respectively, whereas 1000 ng/mL of E2 decreased ($P < 0.05$) progesterone production by 49% (Figure 5A).

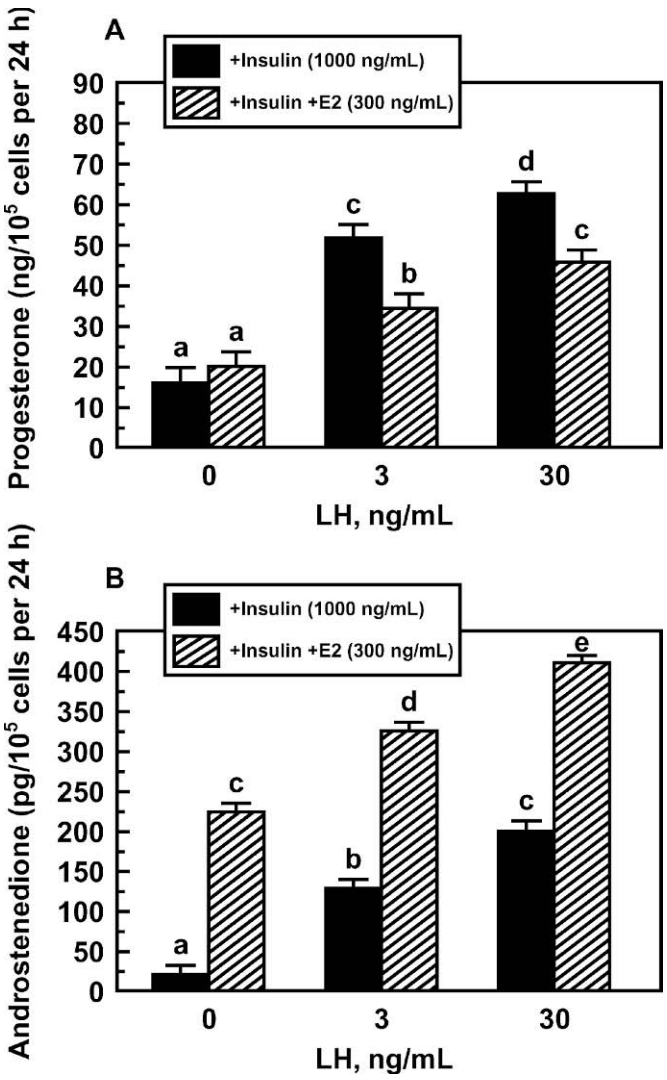

Figure 3. Effect of estradiol (E2) on LH-induced progesterone (panel A) and androstenedione (panel B) production by thecal cells from large follicles (experiment 3). Thecal cells were cultured for 4 d as described in Materials and Methods. During the last 2 d of culture, cells were treated in serum-free medium with 1000 ng/mL of insulin and 0, 3, or 30 ng/mL of LH with or without 300 ng/mL of E2. Values are means from 3 separate experiments. [a,b,c,d,e]Within a panel, means without a common letter differ ($P < 0.05$).

[^{125}I]-IGF-I binding sites. Estradiol had no effect ($P > 0.10$) on the number of [^{125}I]-IGF-I binding sites (Figure 5B).

Cell numbers. Doses of 10 and 100 ng/mL of E2 had no effect ($P > 0.10$) on cell numbers, whereas 1000 ng/mL of E2 weakly decreased (i.e., 11% decrease) cell numbers in the presence of 10 ng/mL of insulin (Table 1).

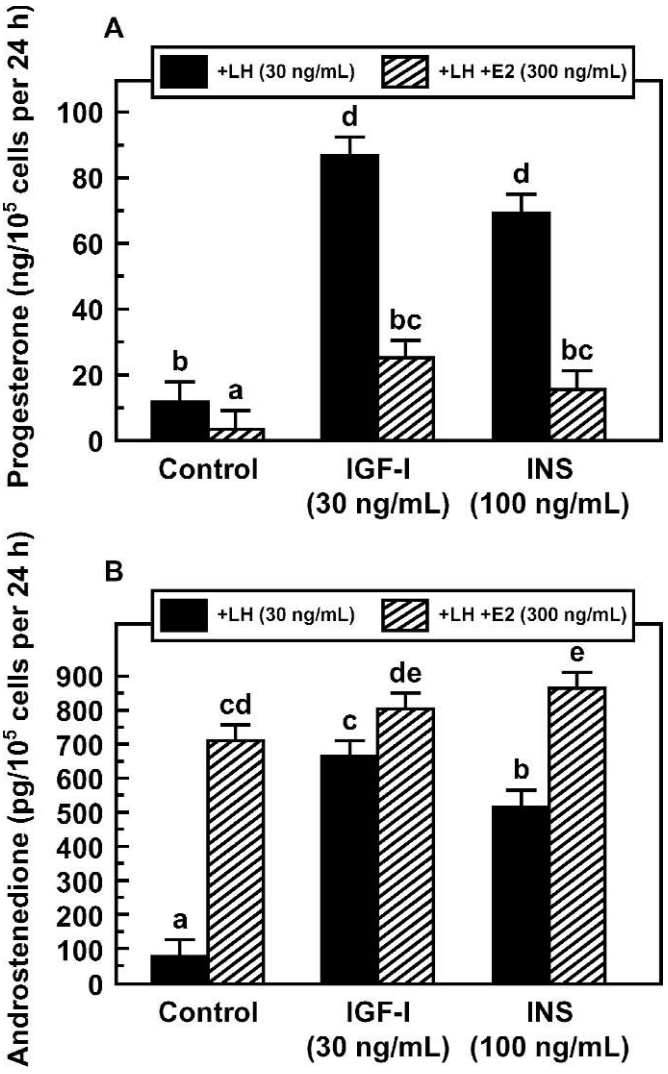

Figure 4. Effect of estradiol (E2) on basal and hormone-induced progesterone (panel A) and androstenedione (panel B) production by thecal cells from large follicles (experiment 4). Thecal cells were cultured for 4 d as described in Materials and Methods. During the last 2 d of culture, cells were treated in serum-free medium with 30 ng/mL of LH, 0 or 100 ng/mL of insulin (INS), and 0 or 30 ng/mL of IGF-I with or without 300 ng/mL of E2. Values are means from 3 separate experiments. [a,b,c,d,e]Within a panel, means without a common letter differ ($P < 0.05$).

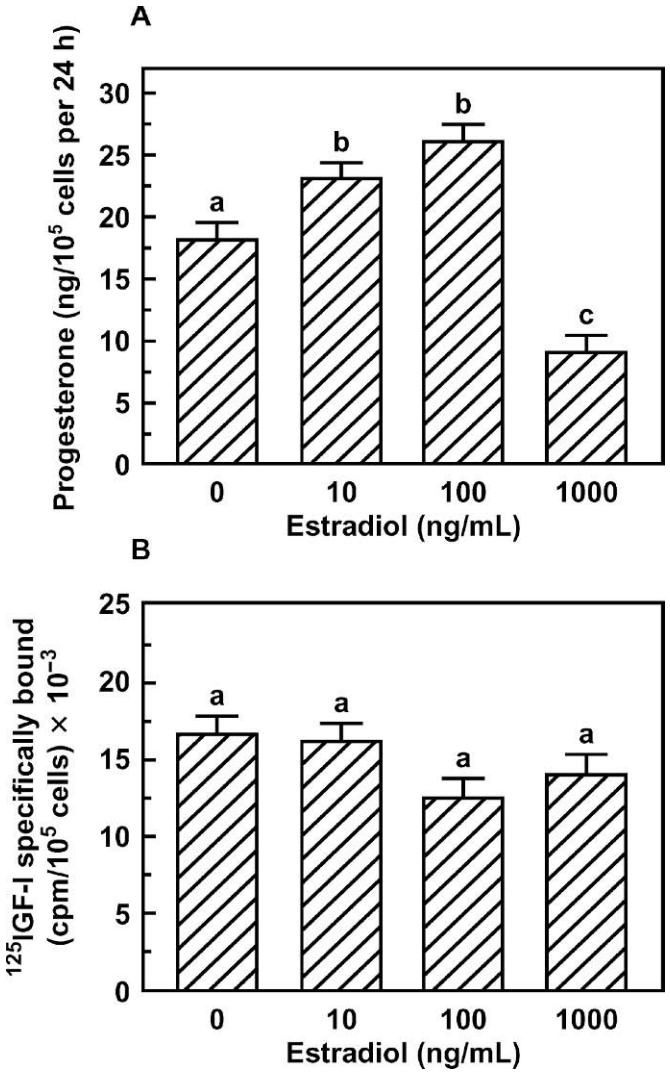

Figure 5. Effect of estradiol (E2) on thecal progesterone production (panel A) and numbers of [^{125}I]-IGF-I binding sites (panel B) (experiment 5). Thecal cells were cultured for 4 d as described in Materials and Methods. During the last 2 d of culture, cells were treated in serum-free medium with 10 ng/mL of insulin with or without the various doses of E2. Values are means from 4 separate experiments. [a,b,c]Within a panel, means without a common letter differ ($P < 0.05$).

DISCUSSION

Results of the present study on bovine thecal cells revealed that: 1) E2 by itself (in the absence of LH) is a weak stimulator of progesterone production by thecal cells; 2) E2 has potent inhibitory effects on thecal cell progesterone production induced by LH, LH plus insulin, and LH plus IGF-I; 3) E2 has potent stimulatory effects on thecal cell androstenedione production in the absence of other hormones as well as in the presence of LH, LH plus IGF-I, and LH plus insulin; 4) E2 has little or no effect on thecal cell numbers; and 5) E2 has no effect on numbers of thecal cell [^{125}I]-IGF-I binding sites.

Previous studies have suggested that E2 either inhibits (Fortune and Hansel, 1979b; Roberts and Skinner, 1990b; Wrathall and Knight, 1995; Gilling-Smith et al., 1997) or has no effect (Tsang et al., 1979; Morley et al., 1989) on progesterone production induced by LH in cultured thecal cells. In the present study, we found that E2 decreased thecal cell progesterone production in the presence of LH alone or a combination of LH and insulin or LH and IGF-I. Similar to previous reports,

LH, insulin, and IGF-I each stimulated progesterone production by bovine thecal cells (Stewart et al., 1995; Spicer and Stewart, 1996a,b; Spicer et al., 1996). In the present study, under basal conditions and in the absence of LH but presence of low (10 ng/mL) concentrations of insulin, E2 (3 to 300 ng/mL) had a weak stimulatory effect on progesterone production. In the presence of FCS, 100 ng/mL to 10 μg/mL of E2 inhibited, whereas 1 and 10 ng/mL of E2 stimulated LH-induced progesterone production by bovine thecal cells (Fortune and Hansel, 1979a,b). Similarly, in serum-free medium, 1 μM (i.e., 272 ng/mL) of E2 inhibited (by 40 to 50%) basal (Roberts and Skinner, 1990a) and human chorionic gonadotropin- (**hCG-**) induced (Roberts and Skinner, 1990b) progesterone production by bovine thecal cells. In rat thecal cells cultured with LH, >500 ng/mL of E2 had no effect on progesterone production but inhibited 17α-hydroxyprogesterone accumulation (Magoffin and Erickson, 1982). In porcine thecal cells, E2 decreased basal and LH-stimulated 17α-hydroxyprogesterone accumulations without affecting progesterone production (Morley et al., 1989), and caused a sustained (2 to 4 d) inhibition of hCG-induced progesterone production (Tonetta et al., 1986). Discrepancies among studies could be due to species differences or differences in culture conditions (e.g., duration of treatment and presence or absence of FCS, insulin, and LH). The normal range of average E2 concentrations in follicular fluid of dairy cattle is from <1 to 1500 ng/mL (Fortune and Hansel, 1979a; Badinga et al., 1992; Stewart et al., 1996; Spicer et al., 2001b). Thus, results of the present and previous studies indicate that at concentrations that mimic intrafollicular levels, E2 may play a major role in the regulation of steroid production by bovine follicles.

Previous studies with bovine (Fortune and Hansel, 1979a; Langhout et al., 1991) and porcine (Veldhuis et al., 1982; Veldhuis, 1985) granulosa cells have also demonstrated species differences in the effect of E2 on steroidogenesis. Specifically, in bovine granulosa cells, E2 inhibits FCS- (Fortune and Hansel, 1979a) and insulin-induced (Langhout et al., 1991) progesterone production. In porcine granulosa cells, E2 enhances basal and gonadotropin-stimulated progesterone production under serum-free (Veldhuis et al., 1984; Veldhuis, 1985; Spicer and Hammond, 1987) and serum-containing (Veldhuis et al., 1982) conditions, but E2 also exhibits biphasic effects on progesterone production (Veldhuis et al., 1981). In particular, this biphasic effect of E2 on progesterone biosynthesis by porcine granulosa cells causes an initial (12 to 24 h) transient inhibition followed by a delayed but sustained (2 to 6 d) stimulation (Veldhuis et al., 1981; Hunter and Armstrong, 1987). The mechanism of action of E2 on progesterone production by porcine granulosa cells appears to be a direct effect on side-chain cleavage enzyme (Veldhuis et al., 1982; Veldhuis, 1985) and 3β-hydroxysteroid dehydrogenase altering conversion of pregnenolone to progesterone (Veldhuis, 1985). Whether this is the point of E2 inhibition on progesterone production by bovine thecal cells in the present study will require further study. Moreover, depletion of putative endogenous pools of progestins and their precursors that are used for androgen production (see next section) could have in part accounted for the decrease in progesterone production caused by E2, but further research will be needed to verify this suggestion.

A stimulatory effect of E2 on androstenedione production has been previously reported for bovine (Roberts and Skinner, 1990a,b; Wrathall and Knight, 1995), human (Gilling-Smith et al., 1997), and porcine (Tsang et al., 1979; Morley et al., 1989) thecal cells, and is confirmed by the present study. We further extend these previous findings, showing that E2 can dramatically enhance basal and LH-induced androstenedione production as well as amplifying insulin-, LH plus insulin-, and LH plus IGF-I-induced androstenedione production. The less than additive androstenedione responses of thecal cells to combined treatments of E2 and insulin or E2 and IGF-I indicate that these hormones may share a common or overlapping intracellular pathway. Alternatively, the 2-d stimulation of thecal cell androstenedione production by E2 may have resulted in depletion of the cholesterol and progestin precursors available for maximal androstenedione production. Thus, the present and previous studies indicate that the inhibitory effect of E2 on progesterone production by bovine thecal cells may be due in part to stimulation of the enzymes that convert progestins into androgens (i.e., stimulation of 17,20 lyase or 17α hydroxylase). Because bovine thecal cells preferentially use the Δ^5-pathway (Lacroix et al., 1974; Fortune, 1986), inhibition of progesterone production by E2 could promote androgen synthesis via the Δ^5-pathway. However, in rats, estrogens inhibit androgen production by thecal cells and inhibit 17α-hydroxyprogesterone accumulation (Magoffin and Erickson, 1981, 1982; Johnson and Crane, 1995) suggesting that E2 inhibits production of important androgen precursors in this species (Liu and Hsueh, 1986). Further research will be needed to identify any shared or overlapping component(s) of the intracellular mechanism by which E2, LH, IGF-I, and insulin stimulate androstenedione production in bovine thecal cells.

No consistent effect of E2 was observed on thecal cell numbers in the present study. Previously, 40-h treatment with estradiol (10 to 1000 ng/mL) has been shown to decrease DNA synthesis (by 20 to 50%) as measured

by [^{3}H]-thymidine incorporation in porcine thecal (Ranson et al., 1997) and granulosa cells (Spicer and Hammond, 1989). Whether the difference between the present and previous studies is due to species differences, duration of treatment, or both will require further study. In nonlactating cows, long-term (i.e., 75 d) E2 treatment increases the number of corpora lutea formed after superovulation in nonlactating cows (Cushman et al., 2001a) and increases percentages of primary follicles (Cushman et al., 2001b). Furthermore, in vitro treatment of bovine preantral follicles with E2 for 7 d decreased numbers of granulosa cells but did not alter their proliferative activity (Hulshof et al., 1995). Thus, additional research will be needed to determine whether E2 directly alters follicle growth in cattle via action on granulosa cells, thecal cells, or both.

In the present study, the mechanism of action of E2 on IGF-I-stimulated progesterone and androstenedione production by bovine thecal cells did not appear to involve a change in IGF type I receptors. In contrast, E2 increases IGF type I receptors in porcine (Veldhuis et al., 1986) and bovine (Spicer et al., 1994) granulosa cells. Thus, differences between granulosa and thecal cells may exist in terms of the mechanism of action of E2 regulating hormone-induced steroidogenesis. This difference in the granulosa and thecal cell responses may be due to differences in the type (i.e., α or β) of estrogen receptor present in granulosa vs. thecal cells, and may prove to be one of the keys to understanding differential E2 responses in these 2 cell types. Previous studies have localized ERα mainly to thecal cells and ERβ mainly to granulosa cells in rat (Sar and Welsch, 1999; Pelletier et al., 2000; Berisha et al., 2002) and bovine (Rosenfeld et al., 1999; Berisha et al., 2002) follicles. In humans, ERα and ERβ are expressed in both granulosa and thecal cells (Saunders et al., 2000; Wang et al., 2000; Jakimiuk et al., 2002). Moreover, estrogen receptors appear to be regulated by hormones because in vivo treatment with hCG/LH dramatically decreases specific [^{3}H]-E2 binding to rat granulosa cells (Richards, 1975) and ERβ mRNA levels in mouse granulosa cells (Byers et al., 1997; Fitzpatrick et al., 1999). Levels of ERα and ERβ mRNA in thecal and granulosa cells also increase with increased follicular size (Manikkam et al., 2001), and follicular E2 levels in cattle (Berisha et al., 2002) and specific binding of [^{3}H]-E2 to rat granulosa cells are increased by E2 treatment in vivo (Richards, 1975). Whether the various hormones used in the present study altered amounts of ERα or ERβ in thecal cells will require further elucidation.

CONCLUSIONS

In conclusion, the present study provides further supportive evidence for a paracrine role of E2 in regulating steroidogenesis of bovine follicles. Our studies indicate that E2 may have a major positive impact on basal, LH-, IGF-I-, and insulin-induced androstenedione production by bovine thecal cells, which could further increase estrogen production by the follicle. Thus, these results support the hypothesis that E2 synthesized within the follicle is part of a paracrine positive feedback system that acts to promote E2 production by the dominant follicle in cattle via increased androstenedione production. Future studies should focus on evaluating the effect of E2 in vivo, so that ultimately, the impact of E2 on reproductive efficiency of dairy cattle can be ascertained.

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