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Influence of Cortisol on Insulin- and Insulin-Like Growth Factor 1 (IGF-1)-Induced Steroid Production and on IGF-1 Receptors in Cultured Bovine Granulosa Cells and Thecal Cells

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During stress, hyperactivity of the adrenal gland can directly and indirectly inhibit ovarian function. However, little evidence existed to support the notion that glucocorticoids could influence insulin-like growth factor 1 (IGF-1) action within the ovary. Therefore, the effect of cortisol on IGF-1-induced granulosa and thecal cell function was evaluated. Granulosa and thecal cells from bovine ovarian follicles were cultured for 2 d in the presence of 10% fetal calf serum and then cultured for an additional 2 d in serum-free medium with added hormones. Cortisol had little or no effect ($p > 0.05$) on IGF-1-induced progesterone production by granulosa cells from both small (1–5 mm) or large (≥8 mm) follicles. Also, cortisol had little or no effect ($p > 0.05$) on basal, insulin-, or IGF-1-induced estradiol production by granulosa cells from small or large follicles, or on the number of IGF-1 receptors in granulosa cells from small follicles. Cortisol had no effect ($p > 0.10$) on insulin-induced granulosa cell numbers, but increased IGF-1-induced granulosa cell numbers. In thecal cells, doses of 1–100 ng/mL of cortisol increased ($p < 0.05$) insulin- and IGF-1-induced thecal cell numbers by 10–20%, progesterone production by 18–36%, and androstenedione production by two- to fourfold. The estimated dose of cortisol necessary to stimulate 50% of the maximum androstenedione production in the presence of IGF-1 was 7 ng/mL. In contrast, cortisol decreased ($p < 0.05$) the number of IGF-1 receptors in thecal cells by 45%. In conclusion, cortisol at physiological levels can directly influence ovarian follicular function in cattle, especially thecal androstenedione production.

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

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Introduction

Exogenous glucocorticoids prolong the reproductive cycles of cattle *(1,2)* and rats *(3,4)*. Moreover, exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induces follicular cysts *(5–7)*. These inhibitory effects of glucocorticoids on reproductive function are owing, in part, to glucocorticoid inhibition of luteinizing hormone (LH) secretion by the anterior pituitary gland *(8–11)*. However, evidence for a direct inhibitory effect of glucocorticoids on ovarian follicular function also exists. For example, in cultured rat granulosa cells, glucocorticoids inhibit follicle-stimulating hormone (FSH)-induced aromatase activity by 50% *(12)* and inhibit the FSH-induced increase in LH receptors *(13)*, the latter of which is a critical step in the development of nonovulatory dominant follicles *(14)* and preovulatory follicles *(15)*. Moreover, the presence of glucocorticoid receptors in homogenates of rat ovaries has been documented *(16)* and provides further support for direct effects of glucocorticoids on ovarian function. However, whether glucocorticoids have direct effects on thecal cell growth and steroidogenesis in any species is unknown. Moreover, evidence for a direct effect of glucocorticoids on granulosa cell function in cattle is meager.

Insulin-like growth factor 1 (IGF-1) and insulin are two hormones present in the circulation as well as in follicular fluid that possesses the ability to regulate both mitogenesis and steroidogenesis of ovarian follicular cells (for review, *see 17*). Because glucocorticoids have been shown to influence the responsiveness of various nonovarian cells to IGF-1 *(18–20)*, we examined: the interaction between cortisol and IGF-1 and insulin on cell numbers and steroidogenesis of bovine granulosa and thecal cells in vitro, and the effect of cortisol on IGF-1 receptor numbers in granulosa and thecal cells.

Results

Influence of Cortisol on Thecal Cell Numbers and Steroidogenesis

Experiments 1 and 2 were conducted to evaluate the dose–response effect of cortisol on the action of LH and

Table 1
Effect of 2-Day Treatment of Various Doses of Cortisol on IGF-1-Induced Cell Proliferation and Progesterone Production in Cultured Granulosa Cells (GC) and Thecal Cells (TC) Collected from Small (S) and Large (L) Bovine Follicles

		Cells/well, $\times 10^5$			Progesterone, ng/10^5 cells/24 h		
Dose of cortisol, ng/mL	Dose of IGF-1, ng/mL	SGC Exp.4	LGC Exp. 5	LTC Exp. 1	SGC Exp. 4	LGC Exp. 5	LTC Exp.1
0	0	0.46^a	0.35^a	1.12^a	35^a	49^a	14^a
0	100	1.11^b	1.17^b	1.34^b	70^b	68^b	43^b
10	100	1.28^c	1.43^d	1.56^c	68^b	59^b	54^c
30	100	1.46^d	$1.39^{c,d}$	1.59^c	77^b	67^b	55^c
100	100	1.26^c	1.16^b	1.62^c	73^b	63^b	52^c
300	100	1.28^c	$1.22^{b,c}$	1.58^c	79^b	76^b	$47^{b,c}$
SEM		0.04	0.07	0.05	6	6	3

^{a,b,c,d}Within a column, means without a common superscript differ ($p < 0.05$). For each experiment, values are means from three separate experiments. For Exp. 1, TC were treated concomitantly with 100 ng/mL of LH. For Exp. 4 and 5, GC were treated concomitantly with 50 ng/mL of FSH.

IGF-1 on thecal cell numbers and steroidogenesis. For Exp. 1, thecal cells from large 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 100 ng/mL of LH in the absence or presence of 100 ng/mL of IGF-1 and cortisol (0, 10, 30, 100, and 300 ng/mL). In the absence of cortisol, 2-d treatment with IGF-1 increased ($p < 0.05$) thecal cell numbers and progesterone production by 1.2- and 3.0-fold, respectively (Exp. 1; Table 1). Two-day treatment with each of the four doses of cortisol increased ($p < 0.05$) IGF-1-induced cell numbers similarly (i.e., 16–21% increases; Table 1). Two-day treatment with all but the 300 ng/mL dose of cortisol increased ($p < 0.05$) IGF-1-induced progesterone production by 22–28% (Exp. 1; Table 1).

Because the significant increase in thecal cell numbers and progesterone induced by cortisol occurred at 10 ng/mL, an additional experiment (Exp. 2) was conducted to determine the effect of lower doses of cortisol on thecal cell function, including androstenedione production. For Exp. 2, thecal cells from large follicles were cultured as described for Exp. 1, except that lower doses of cortisol were tested (i.e., 0, 1, 3, 10, and 30 ng/mL). In the absence of cortisol, 2-d treatment with IGF-1 increased ($p < 0.05$) thecal cell numbers, progesterone production and androstenedione production by 1.7-, 3.1-, and 4.1-fold, respectively (Fig. 1). Treatment with cortisol caused a dose-dependent increase ($p < 0.05$) in IGF-1-induced cell numbers, progesterone production, and androstenedione production (Fig. 1). Maximal stimulation (expressed as percentage increase above controls) of cell numbers, progesterone, and androstenedione production by cortisol averaged 15, 36, and 214%, respectively. The estimated dose of cortisol necessary to stimulate 50% of the maximum (i.e., ED_{50}) cell numbers, progesterone production, and androstenedione production was 4.5, <1, and 7.0 ng/mL, respectively.

Experiment 3 was conducted to compare the influence of cortisol on insulin- and IGF-1-induced thecal cell numbers and steroidogenesis. Thecal cells were cultured as in Exp. 1, except that during the second 2-d culture period, thecal cells were treated with insulin (0 or 100 ng/mL), IGF-1 (0 or 100 ng/mL), and/or cortisol (0 or 30 ng/mL). Both insulin and IGF-1 increased ($p < 0.001$) thecal cell numbers, and progesterone and androstenedione production similarly (Fig. 2). Cortisol further increased insulin- and IGF-1-induced cell numbers ($p < 0.05$), and progesterone ($p < 0.01$) and androstenedione ($p < 0.001$) production (Fig. 2).

Influence of Cortisol on Granulosa Cell Numbers and Progesterone Production

Experiments 4 and 5 were conducted to evaluate the dose–response effect of cortisol on the action of FSH and IGF-1 on cell numbers and progesterone production by granulosa cells collected from small and large follicles, respectively. In Exp. 4, granulosa cells collected from small (1–5 mm) 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 in the absence or presence of 100 ng/mL of IGF-1 and cortisol (0, 10, 30, 100, and 300 ng/mL). In Exp. 5, granulosa cells from large (≥8 mm) follicles were cultured as described for Exp. 4. In the absence of cortisol, 2-d treatment with 100 ng/mL of IGF-1 increased ($p < 0.05$) small-follicle granulosa cell numbers by two- to threefold, and progesterone production by 1.4- to 2.0-fold (Exp. 4; Table 1). At 30 ng/mL, 2-d treatment of cortisol increased ($p < 0.05$) IGF-1-induced cell numbers an additional 19–32%; doses of 10, 100, and 300 ng/mL of cortisol for 2 d also amplified ($p < 0.05$) IGF-1-induced cell numbers, but to a lesser extent as compared with 30 ng/mL (Table 1). In contrast, cortisol did not affect ($p > 0.10$) IGF-1-stimulated progesterone production (Exp. 4; Table 1).

In the absence of cortisol, 2-d treatment with IGF-1 increased ($p < 0.05$) large-follicle granulosa cell numbers and progesterone production by 3.3- and 1.4-fold, respec-

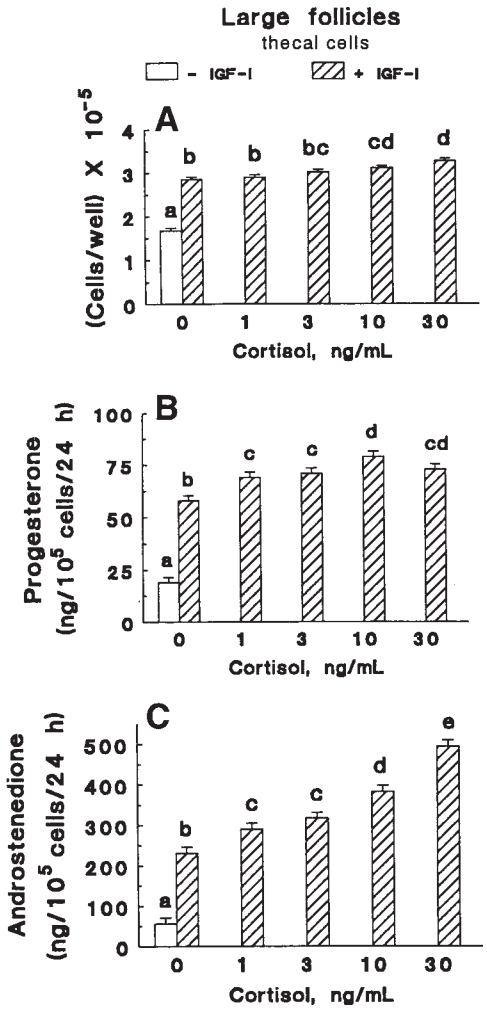

Fig. 1. Effect of cortisol on IGF-1-stimulated cell numbers **(A)**, progesterone **(B)**, and androstenedione **(C)** production by thecal cells of large (≥8 mm) follicles (Exp. 2). Thecal cells from large follicles were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media with 100 ng/mL of LH, and 0 (open bar) or 100 ng/mL (hatched bars) of IGF-1 for an additional 2 d. During the last 2 d of culture, cortisol (0, 1, 3, 10, or 30 ng/mL) was also added to the medium. Values are means of four replicate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Within a panel, means without a common superscript differ ($p < 0.05$).

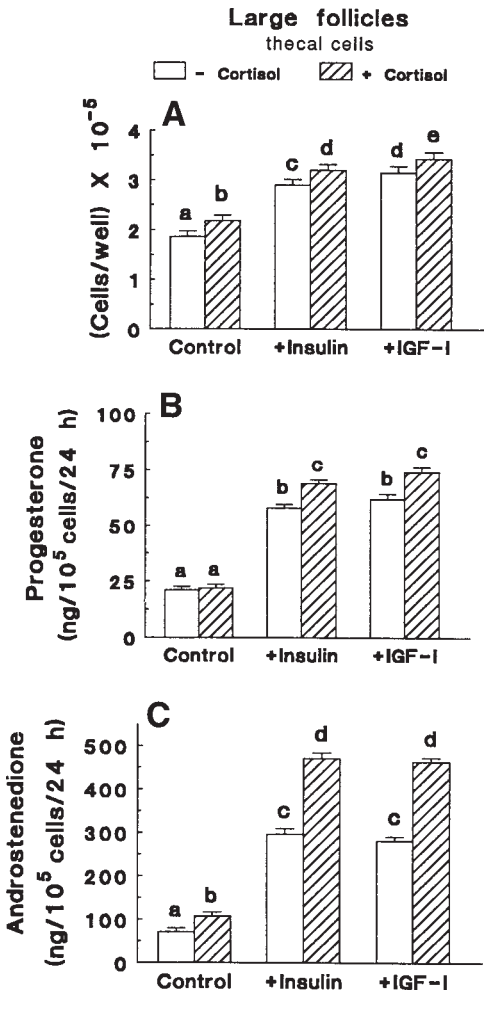

Fig. 2. Effects of cortisol on insulin- and IGF-1-stimulated cell numbers **(A)**, progesterone **(B)**, and androstenedione **(C)** production by thecal cells from large (≥8 mm) follicles (Exp. 3). Thecal cells from large follicles were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media with 100 ng/mL of LH, and 0 (open bar) or 30 ng/mL (hatched bars) of cortisol in the presence or absence of insulin (100 ng/mL) or IGF-1 (100 ng/mL) for an additional 2 d. Values are means from three replicate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Within a panel, means without a common superscript differ ($p < 0.05$).

tively (Exp. 5; Table 1). At 10 and 30 ng/mL, 2-d treatment of cortisol increased ($p < 0.05$) IGF-1-induced cell numbers an additional 19–22% (Table 1). Treatment of 100 and 300 ng/mL of cortisol for 2 d did not significantly affect IGF-1-induced granulosa cells numbers. Similar to granulosa cells from small follicles, 2-d treatment of cortisol did not affect ($p > 0.10$) IGF-1-induced progesterone production by granulosa cells from large follicles (Exp. 5; Table 1).

Influence of Cortisol on Granulosa Cell Aromatase Activity

Experiments 6 and 7 were conducted to evaluate the dose–response effect of cortisol on insulin-induced functional aromatase activity of granulosa cells from small (Exp. 6) and large (Exp. 7) follicles. Granulosa cells from small and large follicles were cultured for 2 d in 10% FCS, and then cultured in serum-free medium for an additional 24 h with 500 ng/mL of testosterone (as an estrogen precursor), 50 ng/mL of FSH, and various doses of cortisol (0, 3, 10, 30, or 100 ng/mL) in the absence or presence of 100 ng/mL of insulin. In the absence of cortisol, 1-d treatment of insulin increased ($p < 0.05$) small- and large-follicle granulosa cell estradiol production by 5.6- and 16.4-fold, respectively, and cell numbers by 2.0- and 1.7-fold, respectively (Table 2). However, 1-d treatment with cortisol had no effect ($p > 0.10$) on insulin-induced cell numbers or estradiol production by

Table 2
Effect of 1-Day Cortisol Treatment on Insulin-Induced Granulosa Cell Numbers and Aromatase Activity of Granulosa Cells Collected from Small (Exp. 6) or Large Follicles (Exp. 7)

		Cells/well, $\times 10^5$		Aromatase activity, ng estradiol/10^5 cells/24 h	
Dose of cortisol	Insulin	Exp. 6	Exp. 7	Exp. 6	Exp. 7
0	0	1.00^a	1.02^a	0.04^a	0.08^a
0	100	1.95^b	1.75^b	0.25^b	1.34^c
3	100	2.07^b	1.64^b	0.26^b	1.61^d
10	100	2.02^b	1.71^b	0.29^b	1.29^c
30	100	2.08^b	1.70^b	0.30^b	$1.44^{c,d}$
100	100	2.02^b	1.68^b	0.21^b	1.02^b
SE		0.04	0.03	0.02	0.08

^{a,b,c,d}Within a column, means without a common superscript differ ($p < 0.05$).

Values are means of three separate replicate experiments in which each treatment was applied in triplicate culture wells; all cells were concomitantly treated with 500 ng/mL of testosterone and 50 ng/mL of FSH.

granulosa cells from small follicles (Exp. 6; Table 2). Insulin-stimulated large-follicle cell numbers were also not affected ($p > 0.10$) by a 1-d treatment of the various doses of cortisol (Exp. 7; Table 2). In contrast to small-follicle granulosa cells, cotreatment with cortisol for 1 d had a biphasic effect ($p < 0.01$) on insulin-induced estradiol production, such that 3 ng/mL of cortisol stimulated (20% increase; $p < 0.05$), 10 and 30 ng/mL of cortisol had no effect ($p > 0.10$), and 100 ng/mL of cortisol inhibited (24% decrease; $p < 0.05$) estradiol production by granulosa cells from large follicles (Exp. 7; Table 2).

Experiment 8 was conducted to compare the effect of cortisol on insulin- and IGF-1-induced granulosa cell aromatase activity. Granulosa cells from small and large follicles were cultured for 2 d in 10% FCS, and then cultured in serum-free medium for an additional 1 or 2 d with 500 ng/mL testosterone (as an estrogen precursor), 50 ng/mL of FSH, cortisol (0 or 30 ng/mL), insulin (0 or 100 ng/mL), and/or IGF-1 (0 or 100 ng/mL). Basal estradiol production by granulosa cells from small follicles did not change ($p > 0.10$) between days 1 and 2. However, estradiol production stimulated by insulin and IGF-1 increased dramatically between days 1 and 2 of treatment (Fig. 3A). After 1 and 2 d of treatment, insulin increased ($p < 0.001$) estradiol production by 2- and 22-fold, respectively; cortisol had no effect ($p > 0.10$) on these insulin responses. After 1 d of treatment, IGF-1 decreased ($p < 0.05$) estradiol production by 72%, whereas after 2 d of treatment, IGF-1 increased ($p < 0.01$) estradiol production by threefold; cortisol had no effect ($p > 0.10$) on these IGF-1 responses (Fig. 3A).

As with small-follicle granulosa cells, basal estradiol production by granulosa cells from large follicles did not change ($p > 0.10$) between days 1 and 2. However, estradiol production stimulated by insulin and IGF-1 increased dramatically between days 1 and 2 of treatment (Fig. 3B). After 1 and 2 d of treatment, insulin increased ($p < 0.001$) estradiol production by 2- and 37-fold, respectively; cortisol had no effect ($p > 0.10$) on these insulin responses.

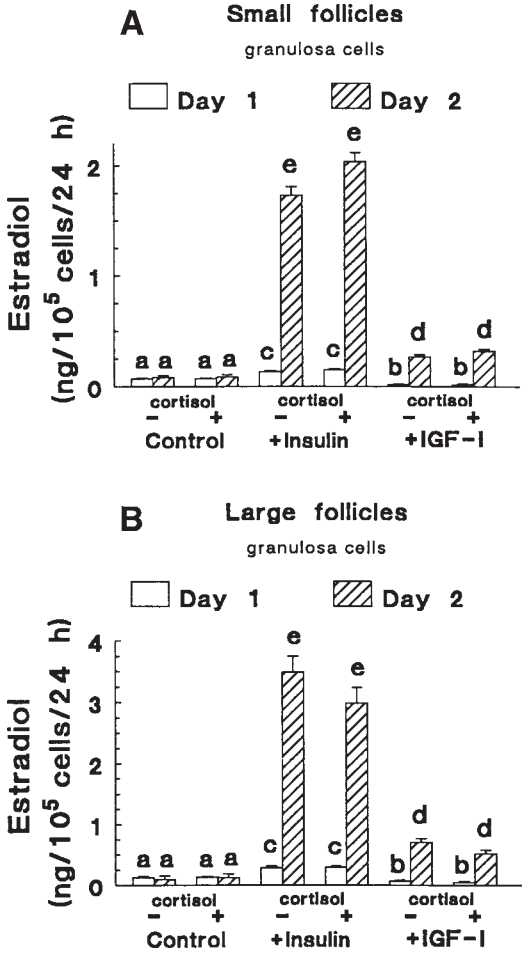

Fig. 3. Effects of cortisol on insulin- and IGF-1-induced estradiol production by granulosa cells in Exp. 8 collected from either small follicles (**A**) or large follicles (**B**). Granulosa cells were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media with 500 ng/mL of testosterone, 50 ng/mL of FSH, cortisol (0 or 30 ng/mL), insulin (0 or 100 ng/mL), and/or IGF-1 (0 or 100 ng/mL) for either 1 d (open bar) or 2 d (hatched bars). Values are means of two replicate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Within a panel, means without a common superscript differ ($p < 0.05$).

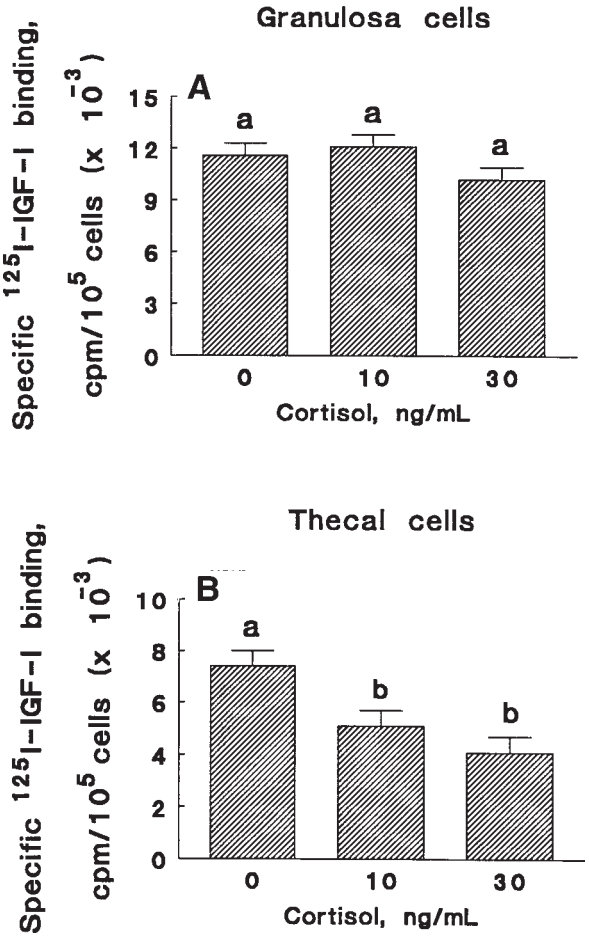

Fig. 4. Effect of cortisol on specific ^{125}I-IGF-1 binding sites in granulosa (Exp. 9; **A**) and thecal (Exp. 10; **B**) cells. Granulosa cells from small follicles and thecal cells from large follicles were cultured for 2 d in the presence of 10% FCS, and then treated in serum-free media with gonadotropin and 10 ng/mL of insulin for an additional 2 d. During the last 2 d of culture, 0, 10, or 30 ng/mL of cortisol were also added to the medium. Values are means of three replicate experiments; within each replicate experiment, each treatment was applied in triplicate culture wells. Within a panel, means without a common superscript differ ($p < 0.05$).

After 1 d of treatment, IGF-1 decreased ($p < 0.05$) estradiol production by 47%, whereas after 2 d of treatment, IGF-1 increased ($p < 0.01$) estradiol production by eightfold; cortisol had no effect ($p > 0.10$) on these IGF-1 responses (Fig. 3B).

Influence of Cortisol on Granulosa and Thecal Cell Receptors for IGF-1

Experiments 9 and 10 were conducted to determine if the positive effect of cortisol on the action of IGF-1 was owing to an increase in numbers of granulosa and thecal cell IGF-1 binding sites, respectively. Granulosa cells from small follicles (Exp. 9) and thecal cells from large follicles (Exp. 10) 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

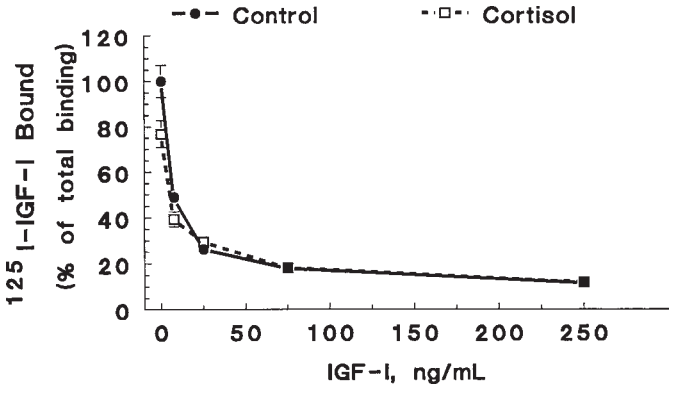

Fig. 5. Effect of cortisol on [^{125}I]-IGF-1 binding to bovine thecal cells. Thecal cells were cultured as described in Fig. 4. During the last 2 d of culture, 0 or 30 ng/mL of cortisol were also added to the medium. Cells were incubated with increasing amounts of unlabeled IGF-1 in the presence of a constant amount of [^{125}I]-IGF-1. Values are means of two replicate experiments; within each replicate, each treatment was applied in triplicate.

FSH (for granulosa cells) or 100 ng/mL of bovine LH (for thecal cells), insulin (10 ng/mL), and cortisol (0, 10, or 30 ng/mL). The doses of gonadotropins and cortisol were selected based on previous studies *(21)* and as per Exp. 1–8. The low dose of insulin was selected in order not to interfere with measurement of IGF-1 binding *(21,22)*. Two-day treatment of 10 or 30 ng/mL of cortisol had no effect ($p > 0.10$) on specific ^{125}I-IGF-1 binding to granulosa cells (Fig. 4A). In contrast, 2-d treatment with 10 and 30 ng/mL of cortisol decreased ($p < 0.05$) specific thecal cell ^{125}I-IGF-1 binding (Fig. 4B). Maximal inhibition (i.e., a 45% decrease) of ^{125}I-IGF-1 binding to thecal cells was observed with 30 ng/mL of cortisol. In this same study, 2-d treatment with 10 and 30 ng/mL of cortisol increased ($p < 0.01$) androstenedione production by 3.5- and 5.2-fold, respectively, and progesterone production by 1.5- and 1.8-fold, respectively. An additional series of experiments using unlabeled IGF-1 competition curves (Fig. 5) and Scatchard analyses *(23)* confirmed that treating thecal cells for 2 d with 30 ng/mL of cortisol reduced the binding capacity and not the dissociation constant of the thecal IGF-1 receptor.

Discussion

Substantial evidence exists to support the notion that increased levels of glucocorticoids in blood may be inhibitory to reproductive functions in heifers and cows *(1,2,5–7,24–27)*. However, no evidence existed previously to support the idea that normal or stress-induced concentrations of cortisol directly influence ovarian cell function in cattle.

In the present study, cortisol, at physiological levels, dramatically enhanced IGF-1- and insulin-stimulated androgen production by thecal cells, an observation not previously reported. Concentrations of cortisol in blood of female cattle under normal and stressful conditions range from 2 to 10 ng/mL *(28–32)* and 10 to 100 ng/mL *(25,26,33)*,

respectively. Average concentrations of cortisol range between 12 and 20 ng/mL in bovine follicular fluid *(32)* and between 31 and 200 ng/mL in human follicular fluid *(34–36)*. Thus, concentrations of cortisol in vivo are within the range of effective doses of the present study. Consistent with our in vitro studies, follicular fluid concentrations of both cortisol and androstenedione decrease with an increase in follicular size in both cyclic and postpartum cattle *(32,37)*. Because cortisol had much weaker effects on IGF-1- and insulin-induced progesterone production than androstenedione production by thecal cells, it is possible that cortisol impacts primarily on thecal cell steroidogenesis by specifically stimulating 17, 20-lyase, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase, or other key enzymes required for thecal androgen synthesis. Further research will be needed to clarify this possibility.

We also observed that physiological concentrations of cortisol had weak stimulatory effects on granulosa and thecal cell numbers, a new finding. Interestingly, low doses (i.e., 10 and 30 ng/mL) were more effective stimulators of IGF-1-induced granulosa cell numbers than were higher doses (i.e., 100 and 300 ng/mL), whereas for thecal cells, doses of >10 ng/mL of cortisol gave similar, but small (<35%) increases in cell numbers. Whether this cortisol-induced increase in cell numbers is owing to increased cell proliferation or increased cell survival is unclear. Previous studies have either not reported effects of cortisol on granulosa cell numbers or found that cortisol had no effect on granulosa cell DNA content *(38)*. In primary rat myoblasts, dexamethasone stimulated cell proliferation *(39,40)*, whereas in human ovarian carcinoma cells, cortisol and dexamethasone at 39 ng/mL inhibited cell growth by 40–50% *(41)*. Cortisol (0.1 μ*M*) also inhibited [^{3}H]-thymidine incorporation in rat calvariae *(42)* and human smooth muscle *(43)*. Reasons for the discrepancies among studies is unclear, but may include differences between species, cell types, or culture conditions. However, results of the present study indicate that cortisol, at physiological levels, may influence follicular growth because of its stimulatory effect on granulosa and thecal cell numbers. In support of this latter suggestion, de Greef and van der Schoot *(44)* reported that dexamethasone treatment in vivo increased the number of healthy follicles on day 9 of pseudopregnancy in rats. In addition, dexamethasone treatment increased the percentage of anovulatory women that ovulated after clomiphene citrate therapy *(45)*, and exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induced follicular cysts *(5–7)*. Whether glucocorticoid treatment alters follicular growth in cattle remains to be determined.

Granulosa cell progesterone production induced by IGF-1 was not influenced by cortisol in the present study. Similarly, cortisol (0.1, 1, or 5 μg/mL) had no effect on FSH-induced progesterone production by porcine granulosa cells cultured in the presence of insulin and 5% pig serum *(46)*, or bovine granulosa cells cultured for 8 d in the presence of FSH and insulin *(38)*. In contrast, cortisol at ≥ 0.1 μ*M* (i.e., ≥36 ng/mL) was found to stimulate progesterone production (not corrected for final cell numbers) by rat granulosa cells cultured for 2 d in serum-free media without insulin or growth factors *(14,47)*, by bovine granulosa cells cultured for 3 d in 10% FCS-containing medium *(48)*, and by human granulosa cells cultured for 6 h in the presence of FSH *(49)*. Because we observed that cortisol potentiated the increase in granulosa cell numbers induced by IGF-1 and previous studies did not correct for final cell numbers, it is possible that the observed increase in progesterone production in some studies was owing to increased cell numbers. Thus, the present study indicates that cortisol has little impact on IGF-1-induced progesterone production by bovine granulosa cells.

We also observed that cortisol (3–100 ng/mL) had little or no effect on IGF-1- or insulin-induced aromatase activity in cultured bovine granulosa cells. Similarly, Kawate et al. *(38)* reported that 8-d treatment with 0.1 μ*M* (i.e., 36 ng/mL) of cortisol had no effect on estradiol production by bovine granulosa cells cultured in the presence of insulin and FSH. In rat granulosa cells cultured in the absence of insulin or growth factors, high concentrations of glucocorticoids have been shown to inhibit FSH-induced aromatase activity *(12,13,47)*. A more recent study indicated that 48-h treatment with 20 n*M* of dexamethasone can increase FSH-induced aromatase activity and mRNA in cultured rat granulosa cells *(50)*. In human granulosa cells, 6-h treatment with 0.1 μ*M* cortisol had no effect on estradiol production whereas 1 μ*M* (i.e., 360 ng/mL) significantly increased estradiol production *(49)*. Reasons for these discrepancies in the effect of corticoids on estradiol production among studies is unknown, but may include differences in duration of treatment, dose and type of glucocorticoid used, and/or the differentiated state of granulosa cells used. The latter two possibilities are supported by our observation that cortisol did not affect estradiol production by small-follicle granulosa cells, whereas cortisol stimulated estradiol production at low doses (i.e., 3 ng/mL) and inhibited estradiol production at high doses (i.e., 100 ng/mL) in large-follicle granulosa cells. Consistent with these in vitro observations, exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induces follicular cysts and decreases estradiol secretion *(5–7)*.

Insulin-induced estradiol production was dramatically increased between days 1 and 2 of treatment, whereas basal estradiol production remained constant. Interestingly, insulin stimulated estradiol production after 1 and 2 d of treatment, whereas IGF-1 inhibited estradiol production after 1 d of treatment and stimulated estradiol production after 2 d of treatment. The stimulatory effect of IGF-1 was only a fraction (i.e., 15–20%) of that observed for insulin. Thus, the present studies along with previous studies *(51–53)* indicate that insulin is a more important stimulator of estra-

diol production by bovine follicles than is IGF-1. In fact, IGF-1 inhibits insulin-induced aromatase activity in bovine granulosa cells *(53)*.

Glucocorticoids have also been shown to inhibit FSH-induced increases in LH receptors (an indicator of granulosa cell differentiation) of rat granulosa cells *(14)*, porcine granulosa cells *(54)*, and bovine granulosa cells *(38)*, but concentrations of cortisol in follicular fluid of cows were not significantly correlated to binding capacity of human chorionic gonadotropin (hCG) in bovine granulosa cells *(32)*. Since high concentrations of glucocorticoids (≥36 ng/mL) were needed to inhibit FSH-induced increases in LH receptors in previous in vitro studies with granulosa cells *(13,38)*, perhaps normally fluctuating levels of glucocorticoids in follicular fluid play no active role in granulosa cell differentiation in vivo. More likely, the evidence to date supports the concept that abnormally high levels of glucocorticoids, manifested only during periods of stress, suppress reproductive activity by directly inhibiting pituitary LH secretion *(8–10)* as well as granulosa cell differentiation *(12,13,47)*.

In the present study, cortisol had no effect on the number of granulosa-cell IGF-1 binding sites, but decreased the number of thecal cell IGF-1 binding sites, a new finding. Thus, it seems that the potentiating effect of cortisol on IGF-1-induced granulosa and thecal cell proliferation and steroidogenesis is not mediated by an increase in the number of IGF-1 binding sites. Our results are consistent with a previous report showing that 1- to 3-d treatment with 1 μ*M* of dexamethasone decreased type 1 IGF receptor mRNA in cultured whole ovarian dispersates *(55)*. In contrast, other studies have shown that dexamethasone can increase IGF-1 binding by human fibroblasts *(18,19)* and rat skeletal muscle cells *(20)*. In human breast cancer cells, dexamethasone has no effect on IGF-1 receptor content *(56)*. Thus, it appears that the effect of glucocorticoids on IGF-1 receptors may depend on the type of cell. The mechanism whereby cortisol potentiates the effect of IGF-1 on granulosa and thecal cell numbers and steroidogenesis will require further elucidation.

Materials and Methods

Reagents and Hormones

Reagents were Dulbecco's Modified Eagle Medium (DMEM), Ham's F12, insulin (bovine; 25.7 U/mg), cortisol, progesterone, enzymes, and FCS, all obtained from Sigma Chemical Co. (St. Louis, MO); ovine FSH (F1913, FSH activity 15 × NIH-FSH-S1 U/mg) and bovine LH (L1914, LH activity 2.0 × NIH-LH-S1 U/mg) obtained from Scripps Laboratories (San Diego, CA); testosterone was obtained from Steraloids (Wilton, NH); and IGF-1 (recombinant human) was from R&D Systems (Minneapolis, MN).

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), granulosa cells from small (1–5 mm) and large (≥8 mm) follicles were collected by aspiration using a needle and syringe, and then washed twice in serum-free medium as described previously *(57)*. Thecal cells were obtained from the ovaries in a similar manner as previously described *(21,58)*. Briefly, large (≥8 mm) follicles that appeared healthy (i.e., well vascularized and having transparent follicular fluid) were dissected from the ovary, and follicular fluid aspirated. Follicles were then bisected, the theca interna layer was microdissected from the follicle wall, granulosa cells were removed, and the theca was enzymatically digested for 1 h at 37°C. 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 (i.e., <10%; *51,58*). Cells were resuspended in serum-free medium, and the number of viable cells was determined using the trypan blue exclusion method. Cell viability averaged 25 ± 4, 29 ± 6, and 89 ± 3% for small-follicle granulosa, large-follicle granulosa, and thecal cells, respectively, at the time of plating.

Medium was a 1:1 (vol/vol) 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 15–105 μL of medium were added to Falcon 24-well plates (#3047; Becton Dickinson and Co., 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. After this time, cells were washed twice with 0.5 mL of serum-free medium and incubations continued in serum-free medium with or without added hormones. Medium was changed every day. For experiments evaluating the effects of hormones on steroid production, hormonal treatments were applied for an additional 2 d (i.e., from days 2–4 of culture), unless stated otherwise.

Determination of Granulosa and Thecal Cell Numbers

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 *(21,57)*. 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, 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 kits (ICN Biomedicals, Costa Mesa, CA) as previously described *(21)*. Intra- and inter-assay coefficients of variation were 8 and 16%, respec-

tively. Sensitivity of the androstenedione assay was 33 pg/mL. Crossreactivity of cortisol in the androstenedione assay was < 0.001%, and thus, concentrations of cortisol used in the present study did not interfere with the androstenedione radioimmunoassay.

Progesterone Radioimmunoassay

Concentrations of progesterone in culture medium collected on day 4 of culture were determined with a radioimmunoassay as previously described *(21,57)*. Intra- and interassay coefficients of variation were 11 and 18%, respectively. Sensitivity of the progesterone assay was 250 pg/mL. Crossreactivity of cortisol in the progesterone assay was < 0.05%, and thus, concentrations of cortisol used in the present study did not interfere with the progesterone radioimmunoassay.

Functional Aromatase Activity

Functional aromatase activity was assessed during the last 24 h of exposure of granulosa cells to testosterone as described for Exp. 6, 7, and 8. After the last 24 h of incubation, concentrations of estradiol in medium were determined by radioimmunoassay *(52),* and cell numbers determined. Estradiol production was expressed as ng/10^5 cells/24 h. Intra- and interassay coefficients of variation were 7 and 19%, respectively. Sensitivity of the estradiol assay was 5 pg/mL. Crossreactivity of cortisol in the estradiol assay was < 0.00001%, and thus, concentrations of cortisol used in the present study did not interfere in the estradiol radioimmunoassay.

^{125}I-IGF-1 Receptor Assay

Specific binding of ^{125}I-IGF-1 to granulosa and thecal cells was determined as previously described *(21,22)*. Briefly, cells were incubated with a saturating amount (i.e., 200,00 cpm/well) of ^{125}I-IGF-1 directly in the 17-mm wells for 16 h at 4°C. At the end of incubation, wells were washed twice with 0.5 mL of 0.15 *M* saline. Cells were then solubilized with 0.25 mL of 1 *N* NaOH and counted in an automated γ-counter (counter efficiency = 75%). Total binding and nonspecific binding (i.e., in the presence of 0.25 μg IGF-1) were quantified using duplicate determinations.

Statistical Analyses

Experimental data are presented as the least-squares means ± SE of measurements for triplicate culture wells from two or more replicated experiments. Each replicate experiment was performed with different pools of granulosa and thecal cells collected from 10–60 ovaries (X ± SE, 21 ± 2 ovaries) for each pool. Main effects (e.g., dose) and interactions on dependent variables (i.e., steroid production) were assessed using general linear models procedure of SAS *(59)*. Each well was considered an experimental unit, and each replicated experiment contained three experimental units per treatment. When steroid production was expressed as ng or pg/10^5 cells/24 h, cell numbers at the termination of the experiment were used for this calculation. Specific mean differences among treatments were determined using the Fisher's protected least-significant difference procedure *(60)*.

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