

Relationship between Concentrations of Immunoreactive Insulin-Like Growth Factor-I in Follicular Fluid and Various Biochemical Markers of Differentiation in Bovine Antral Follicles

L. J. SPICER,² S. E. ECHTERNKAMP,³ S. F. CANNING,² and J. M. HAMMOND^{1,2}

Department of Medicine²
Division of Endocrinology
The Milton S. Hershey Medical Center
The Pennsylvania State University
Hershey, Pennsylvania 17033

and

United States Department of Agriculture³
Roman L. Hruska U. S. Meat Animal Research Center
Clay Center, Nebraska 68933

ABSTRACT

Three experiments were conducted to determine the relationship between concentrations of insulin-like growth factor-I (IGF-I) in ovarian follicular fluid and various biochemical markers of follicular differentiation in bovine follicles. In Experiment I, ovaries were removed on Days 7, 14, 28, 42, or 56 after parturition from a total of 21 cows. In Experiment II, ovaries of 31 cows were removed between Days 20 and 30 postpartum after 48 or 96 h of either saline (0.9% NaCl, 5 ml) or luteinizing hormone-releasing hormone (LHRH, 500 ng/5 ml saline) injections given every 2 h via jugular cannulae. In Experiment III, ovaries of six cows were removed 48-50 h after a 35-mg injection of prostaglandin $F_{2\alpha}$ during the midluteal phase of an estrous cycle. In Experiments I and II, all follicles > 8.0 mm in diameter were removed from each ovary ($n = 33$ and 46 , respectively). In Experiment III, fluid from all follicles > 4 mm in diameter were removed individually ($n = 10$), and fluid from follicles 1-4 mm in diameter were pooled for each cow. Follicles for each experiment were further categorized as either estrogen-active (E-A, concentration of estradiol $>$ progesterone in follicular fluid) or estrogen-inactive (E-I, concentration of progesterone $>$ estradiol in follicular fluid). Measurements of immunoreactive IGF-I (i-IGF-I) were made after separating IGFs from their binding proteins with an acid-ethanol extraction. Levels of i-IGF-I in follicular fluid (72-149 ng/ml) did not significantly change with time postpartum (Experiment I) nor change in response to LHRH injections (Experiment II). In Experiment III, concentrations of i-IGF-I in fluid of 1-4 mm, 5-12-mm or > 12 -mm follicles did not differ ($p > 0.10$). Although E-A follicles contained 15- to 61-fold greater levels of estradiol than E-I follicles across all three experiments, i-IGF-I levels did not differ ($p > 0.10$) between E-A and E-I follicles. Also, in Experiments I and II, levels of androstenedione in follicular fluid and numbers of granulosa cell human chorionic gonadotropin (hCG) binding sites were not significantly correlated with levels of i-IGF-I in follicular fluid. However, a positive correlation was found between concentrations of i-IGF-I (range 11-248 ng/ml) and progesterone (range 19-1500 ng/ml) in fluid in E-I (but not E-A) follicles of Experiments I and II ($r = 0.56$, $p < 0.05$, and $r = 0.82$, $p < 0.01$, respectively). Levels of i-IGF-I in follicular fluid were also positively correlated with follicular diameter in E-A follicles of Experiment I ($r = 0.62$, $p < 0.05$) and III ($r = 0.72$, $p < 0.05$). Levels of i-IGF-I were negatively correlated with numbers of granulosa cell follicle-stimulating hormone binding sites ($r = -0.54$, $p < 0.01$), numbers of thecal hCG binding sites ($r = -0.33$, $p < 0.05$), and levels of estradiol ($r = -0.45$, $p < 0.01$) in E-I follicles of Experiment II. In summary, follicular differentiation in cattle accompanied by dramatic increases in follicular fluid levels of estradiol occurred without changes in levels of i-IGF-I. In contrast, increased concentrations of progesterone in follicular fluid were associated with increased levels of i-IGF-I in individual follicles. Thus, in contrast to results from *in vitro* studies, it seems unlikely that concentrations of IGF-I in follicular fluid are limiting for ovarian follicular estradiol production *in vivo*. However, results of the present study are consistent with the hypothesis that IGF-I levels in follicular fluid may regulate progesterone production *in vivo*.

Accepted May 9, 1988.
Received September 24, 1987.

¹ Reprint requests: James M. Hammond, M.D., Department of Medicine/Endocrinology, The Milton S. Hershey Medical Center, The Pennsylvania State University, P. O. Box 850, Hershey, PA 17033.

INTRODUCTION

Previously, *in vitro* studies have established the ovarian granulosa cell as a site of insulin-like growth factor-I (IGF-I) action and secretion (Baranao and Hammond, 1984; Adashi et al., 1985b,c; Davoren et al., 1985; Hammond et al., 1985; Veldhuis et al., 1985a,b). Specifically, IGF-I (i.e., Somatomedin-C) has been shown to enhance follicle-stimulating hormone (FSH)-stimulated estrogen and/or basal and FSH-stimulated progesterin production in murine (Adashi et al., 1985a,b,c; Davoren et al., 1985; 1986), bovine (Schams, 1987), and porcine granulosa cells (Baranao and Hammond, 1984; Veldhuis et al., 1985a,b; 1986a,b; 1987a,b; Maruo et al., 1988). In addition, the presence of high-affinity, low-capacity binding sites for IGF-I in granulosa cells of rats (Adashi et al., 1986, 1988b; Davoren et al., 1986) and pigs (Baranao and Hammond, 1984; Veldhuis et al., 1985b; Maruo et al., 1988) has been documented. More recently, the production of an immunoreactive IGF-I (i-IGF-I) by porcine granulosa cells *in vitro* has been reported (Hammond et al., 1985) and appears to be under hormonal (i.e., luteinizing hormone [LH], FSH, growth hormone [GH], and estradiol) control (Hsu and Hammond, 1987a,b). Although i-IGF-I levels in porcine follicular fluid increase with increased follicular size (Hammond et al., 1985; 1988), no other evidence has been reported supporting the notion that fluctuating levels of IGF-I regulate follicular differentiation *in vivo*.

Therefore, we measured concentrations of i-IGF-I, progesterone, androstenedione, and estradiol (E_2) in follicular fluid, and numbers of gonadotropin receptors in the same follicles collected in two experiments. Previously, we have reported from these same studies that the steroidogenic capacity of large (≥ 8 mm) follicles increase with time postpartum (Spicer et al., 1986a) and in response to low-dose injections of luteinizing hormone-releasing hormone (LHRH) (Spicer et al., 1986b) in anovulatory cattle. Thus, in Experiments I and II, IGF-I levels were measured in follicular fluid previously assayed for steroids (Spicer et al., 1986a,b) to determine if i-IGF-I levels in fluid of large follicles changed with time postpartum or in response to low-dose injections of LHRH. A third experiment was conducted to determine if i-IGF-I levels in fluid of antral follicles were associated with follicular diameter or concentrations of E_2 , or progesterone in the same follicles collected during the preovulatory period of an estrous cycle in cattle.

MATERIALS AND METHODS

Experiment I

Over an 8-wk period (July to September), 32 primiparous, suckled beef cows were slaughtered on either Day 7 ($n = 6$), 14 ($n = 6$), 28 ($n = 6$), 42 ($n = 8$), or 56 ($n = 6$) after parturition as previously reported (Spicer et al., 1986d). Only the anovulatory cows (as determined by absence of newly formed corpora lutea and/or serum progesterone < 1 ng/ml) were used in the present study. The number of anovulatory cows on Days 7, 14, 28, 42, and 56 were 6, 6, 5, 3, and 1, respectively. Within 30 min of slaughter, ovaries were removed and placed on ice; all follicles ≥ 8.0 mm in diameter were removed from each pair of ovaries. Follicles were dissected free of ovarian stroma, diameters were recorded, and follicular fluid was collected as described (Spicer et al., 1986a). Follicular fluid was stored frozen at -20°C until concentrations of progesterone, androstenedione, and E_2 were determined by radioimmunoassay as previously described (Ireland and Roche, 1982; Spicer et al., 1986a).

Immunoreactive IGF-I (i-IGF-I) in follicular fluid was determined by radioimmunoassay after an acid-ethanol extraction to dissociate IGFs from their binding protein as previously described (Hammond et al., 1985; 1988). Briefly, aliquots of follicular fluid were diluted 1:4 with 87.5% acidic ethanol (0.25 N HCl final concentration) and incubated overnight at 4°C . The samples were then centrifuged for 30 min at $1200 \times g$ at 4°C . After neutralization with 0.855 M tris(hydroxymethyl)aminomethane (TRIS), an aliquot of the supernatant was then used in the radioimmunoassay. This extraction procedure resulted in parallelism between the IGF-I standard and bovine follicular fluid (Fig. 1). The IGF-I intra- and interassay coefficients of variation were 5.3 and 8.4%, respectively. Stability of i-IGF-I in follicular fluid was evaluated by freeze-thawing a pool of bovine follicular fluid 1, 3, 9, or 27 times. Three, 9, or 27 freeze-thaw cycles (vs. 1 freeze-thaw cycle) had no significant effect on the concentration of i-IGF-I. The concentration of i-IGF-I after 3, 9, and 27 freeze-thaw cycles was 97, 98, and 98%, respectively, of that measured after one freeze-thaw. Similarly, Furlanetto and Marino (1987) have reported that serum exposed to 9 repetitive freeze-thaw cycles did not alter i-IGF-I levels.

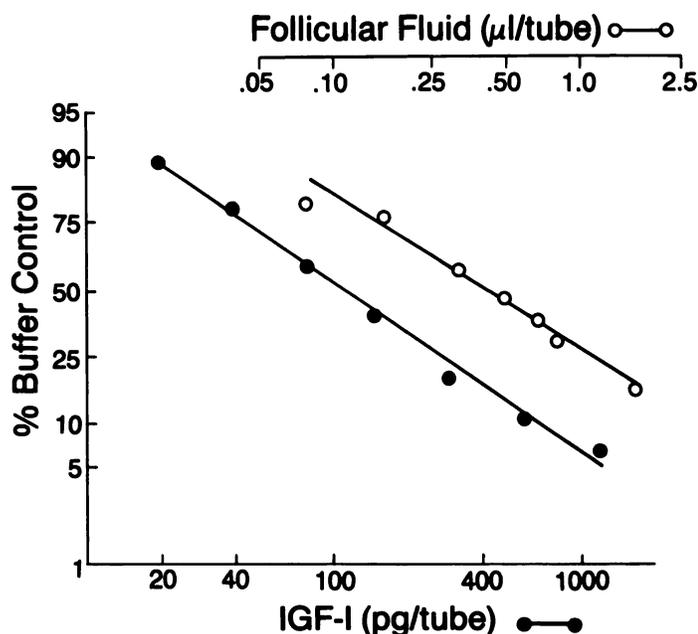


FIG. 1. Competition of acid-ethanol-treated bovine follicular fluid in the insulin-like growth factor-I (IGF-I) radioimmunoassay. A pool of follicular fluid from >8 mm follicles collected from postpartum cattle was treated with acid-ethanol as described in *Materials and Methods*. Increasing volumes of this follicular fluid extract were lyophilized and reconstituted in assay buffer and expressed in μ l follicular fluid equivalents added per tube as percentage of total binding (Buffer Control) of [125 I]iodo-IGF-I (Amgen Biologicals, Thousand Oaks, CA) on a log-logit plot. Displacement curve for authentic IGF-I (Amgen Biologicals) is also shown.

Granulosa and thecal layers were removed and separated via blunt dissection, quickly frozen, and stored at -70°C in phosphate-buffered saline (PBS): 20% glycerol (vol:vol) until numbers of human chorionic gonadotropin (hCG) and follicle-stimulating hormone (FSH) binding sites (expressed as dpm/ μ g DNA) were determined by saturation analysis as previously described (Ireland and Roche, 1982; Spicer et al., 1986a; Spicer and Ireland, 1986). The distribution of numbers of cows and follicles processed in each treatment group is listed in Table 1. Of cows that had initiated estrous cyclicity, first ovulation occurred 41 ± 1 days after parturition (Spicer et al., 1986d).

Analysis of variance, with "days after parturition" as the main effect, was used to determine changes in levels of follicular i-IGF-I, steroids, or gonadotropin binding. Data from cows on Days 42 and 56 were combined to form a group identified as Days 42–56 ($n = 4$). Results of analysis of variance were similar whether "cow" or "follicle" was used as the replicate

TABLE 1. Distribution of numbers of cows and follicles processed for Experiments I and II.

Experimental group	No. of cows ^a	No. of follicles	
		Total	Range/cow
Experiment I			
Day 7	6	9	1–2
Day 14	6	10	1–3
Day 28	5	8	1–3
Day 42–56	4	6	1–2
Experiment II			
48 h saline	6	9	1–2
96 h saline	7	9	1–4
48 h LHRH*	9	16	1–3
96 h LHRH	9	12	1–2

^aTotal number of cows in each experimental group from which follicles were collected.

*LHRH = luteinizing hormone-releasing hormone.

within experimental groups. Differences between means were determined using Fisher's Protected Least-Significant mean test (Ott, 1977). In addition, i-IGF-I levels were evaluated after follicles were categorized as estrogen-active (E-A; concentrations of $\text{E}_2 >$ progesterone in follicular fluid) or estrogen-inactive (E-I; concentrations of progesterone $>$ E_2 in follicular fluid), since E-A follicles in cattle are predominantly healthy (nonatretic) follicles (Ireland and Roche, 1982). Student's *t*-test was used for comparisons of two means.

Experiment II

Over a 3-wk period (September to October), 31 pluriparous, suckled beef cows were allotted to one of four treatment groups as described (Spicer et al., 1986c). All cows used in the present study were anovulatory on the basis of absence of newly formed corpora lutea and/or serum progesterone <1 ng/ml. Briefly, treatments were begun on Day 21 after parturition and were 1) injections of saline for 48 h, $n = 6$; 2) injections of saline for 96 h, $n = 7$; 3) injections of LHRH for 48 h, $n = 9$; and 4) injections of LHRH for 96 h, $n = 9$. Saline (0.9% NaCl, 5 ml) and LHRH (Beckman, Palo Alto, CA; 500 ng/5 ml saline) were injected i.v. at 2-h intervals via a jugular cannula. Two hours after the last injection, all cows were ovariectomized under local anesthesia (2% procaine HCl) by inserting a 50-cm serrated spay scissors through an incision in the dorsal wall of the vagina. Immediately after ovariectomy, ovaries were placed on ice and processed as described for Experiment I.

Concentrations of progesterone, androstenedione, E_2 , and i-IGF-I in follicular fluid, and numbers of hCG (LH) and FSH binding sites were quantified as in Experiment I. The distribution of numbers of cows and follicles processed in each treatment group is listed in Table 1. LHRH treatment induced LH and FSH pulses in all cattle but did not induce ovulation in any of the cows within the 96-h period studied (Spicer et al., 1986c).

Data were subjected to factorial analysis of variance with "LHRH" and "interval of injection" as main effects and interactions to determine changes in levels of follicular fluid i-IGF-I, steroids, or gonadotropin binding. Mean differences were determined using Fisher's Protected Least Significant Difference mean test (Ott, 1977). As in Experiment I, i-IGF-I levels were evaluated after follicles were categorized as either E-A or E-I. Student's *t*-test was used for comparisons of two means.

Experiment III

Six pluriparous beef cows exhibiting regular estrous cycles were synchronized with a 35-mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; lutalyse, Upjohn Co., Kalamazoo, MI) injection (i.m.) during the midluteal phase. Ovaries were collected 48–50 h after $PGF_{2\alpha}$. Ovariectomies were performed as described in Experiment II. Blood was collected for progesterone determinations to verify that luteal regression had occurred. Immediately after ovariectomy, ovaries were placed on ice. The surface diameter of all follicles >4 mm were recorded and their follicular fluid was collected individually. Follicular fluid from all follicles 1–4 mm in diameter were collected and pooled for each cow. Follicular fluid was stored at -20°C until concentrations of i-IGF-I, E_2 , and progesterone were quantified as in Experiment I.

Data were grouped into three sizes (1–4 mm, 5–12 mm, and >12 mm) and subjected to analysis of variance with "size of follicle" as the main effect to determine if i-IGF-I or steroids in follicular fluid varied with follicular size. In addition, i-IGF-I levels were evaluated after follicles were categorized as either E-A or E-I as in Experiments I and II. Student's *t*-test was used for comparisons of two means.

RESULTS

Experiment I

Concentrations of i-IGF-I in follicular fluid of large (>8 mm) follicles did not change ($p>0.10$) with time

postpartum; i-IGF-I averaged 139 ± 13 , 137 ± 13 , 143 ± 10 , and 147 ± 15 on Days 7, 14, 28, and 42–56, respectively. In contrast, concentrations of E_2 in fluid of these same follicles increased from 48 ± 24 ng/ml to 273 ± 83 ng/ml between Days 7 and 28 postpartum ($p<0.05$). Likewise, follicles classified as E-A contained 28-fold greater concentrations of E_2 than E-I follicles ($p<0.01$) whereas i-IGF-I levels did not differ between E-A and E-I follicles ($p>0.10$) (Table 2). Progesterone levels were 2-fold greater in E-I than in E-A follicles ($p<0.05$). Concentrations of androstenedione (Table 2), numbers of [^{125}I]iodo-hCG binding sites in thecal cells (average = 337 ± 56 dpm/ μg DNA) and granulosa cells (average = 878 ± 165 dpm/ μg DNA), and numbers of [^{125}I]iodo-FSH binding sites in granulosa cells (Table 2) did not differ between E-A and E-I follicles. However, concentrations of i-IGF-I were correlated with progesterone levels in E-I follicles ($r = 0.56$, Fig. 2A), and with

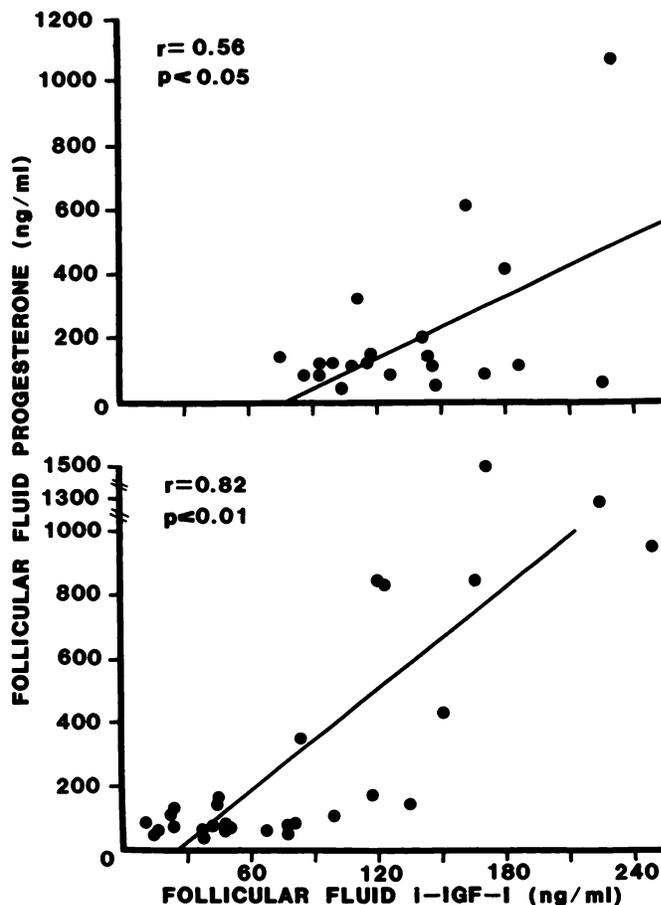


FIG. 2. The relationship between concentrations of immunoreactive insulin-like growth factor-I (i-IGF-I) and progesterone in follicular fluid of estrogen-inactive (E-I) follicles in anovulatory cattle from Experiment I ($n = 21$, top panel) and Experiment II ($n = 29$, bottom panel).

TABLE 2. Concentrations of immunoreactive insulin-like growth factor-I (i-IGF-I), estradiol (E_2), progesterone (P), and androstenedione (A) in follicular fluid and numbers of [125 I]iodo-follicle-stimulating hormone (FSH) binding sites in granulosa cells of large follicles categorized as either estrogen-active (E-A) or estrogen-inactive (E-I).^a

	(n) ^b	Average diameter (mm)	Hormone concentration (ng/ml follicular fluid)				[125 I]iodo-FSH (dpm/ μ g DNA)
			i-IGF-I	E_2	P	A	
Experiment I							
E-A	12	11.6 \pm 0.4 ^c	149 \pm 7	338 \pm 45 ^c	96 \pm 5 ^c	14 \pm 4	574 \pm 41
E-I	21	9.2 \pm 0.3 ^d	136 \pm 9	12 \pm 3 ^d	197 \pm 53 ^d	18 \pm 3	644 \pm 93
(range)		(8.0–14.2)	(75–230)	(1–606)	(38–1065)	(4–49)	(139–1652)
Experiment II							
E-A	17	11.3 \pm 0.6	72 \pm 12	180 \pm 25 ^c	60 \pm 6 ^c	19 \pm 5	1364 \pm 223 ^c
E-I	29	9.9 \pm 0.4	81 \pm 11	12 \pm 3 ^d	333 \pm 79 ^d	16 \pm 3	933 \pm 166 ^d
(range)		(8.0–15.3)	(11–248)	(1–379)	(19–1500)	(1–69)	(109–3602)
Experiment III							
E-A	7	12.1 \pm 1.4	146 \pm 28	680 \pm 92 ^c	47 \pm 8 ^c	ND ^e	ND
E-I	3	9.3 \pm 1.5	181 \pm 35	16 \pm 5 ^d	217 \pm 46 ^d	ND	ND
(range)		(6–19)	(50–292)	(3–1021)	(25–274)		

^aE-A = E_2 concentration > P concentration in follicular fluid; E-I = P concentration > E_2 concentration in follicular fluid. Values are means \pm SE.

^bNumber of follicles.

^{c,d}For each experiment, means within a column with different superscripts differ ($p < 0.05$).

^eND, Not determined.

follicular diameter ($r = 0.62$) and numbers of LH binding sites in granulosa cells of E-A follicles ($r = 0.46$) (Table 3). Other correlations were not significant ($p > 0.10$, see Table 3).

Experiment II

LHRH treatment or interval of injections did not affect concentrations of I-IGF-I in follicular fluid of large (≥ 8 mm) follicles, i-IGF-I averaged 83 ± 12

and 70 ± 12 ng/ml in LHRH- and saline-treated cows, respectively. Therefore, all follicles were classified as either E-A or E-I as described in Experiment I (see Table 2). Although concentrations of E_2 were 15-fold greater in E-A than in E-I follicles, i-IGF-I levels were similar ($p > 0.10$). Progesterone levels were nearly 6-fold greater in E-I than in E-A follicles ($p < 0.05$). Concentrations of androstenedione (Table 2), and numbers of [125 I]iodo-hCG binding sites in thecal cells (average = 443 ± 88 dpm/ μ g DNA) and granulosa

TABLE 3. Correlations between concentrations of insulin-like growth factor-I (IGF-I) in follicular fluid and progesterone (P), androstenedione (A), and estradiol (E_2), concentrations in follicular fluid, follicular diameter, and numbers of gonadotropin receptors (R) in granulosa cells (GC) and theca (T).

	(n)	Diameter	P	A	E_2	GC-LH-R	GC-FSH-R	T-LH-R	
Experiment I									
IGF-I	E-A ^a	12	0.62**	-0.34	-0.02	0.19	0.46*	-0.12	-0.31
	E-I ^b	21	0.04	0.56***	-0.30	0.11	-0.07	0.21	0.26
Experiment II									
IGF-I	E-A	17	0.23	0.34*	0.20	0.10	0.13	-0.29	-0.13
	E-I	30	0.04	0.82***	-0.14	-0.45***	0.28*	-0.54***	-0.33**
Experiment III									
IGF-I	E-A	7	0.72**	0.56*	ND ^c	-0.57*	ND	ND	ND

* $p < 0.10$.

** $p < 0.05$.

*** $p < 0.01$.

^aE-A = Estrogen-active.

^bE-I = Estrogen-inactive.

^cND = Not determined.

TABLE 4. Concentrations of immunoreactive insulin-like growth factor-I (i-IGF-I), estradiol (E_2) and progesterone (P) in follicular fluid of various-sized follicles in Experiment III.

Diameter	(n) ^a	Hormone concentration (ng/ml follicular fluid)		
		i-IGF-I	E_2	P
<4 mm	6	170 ± 30	25 ± 18 ^b	54 ± 12
5–12 mm	5	156 ± 32	312 ± 135 ^c	132 ± 52
>12 mm	5	156 ± 32	650 ± 159 ^c	64 ± 13

^aNumber of cows for <4 mm group, and number of individual follicles for 5–12 mm and >12 mm groups.

^{b,c}Means within a column with different superscripts differ ($p < 0.05$).

cells (average = 1446 ± 240 dpm/ μ g DNA) did not differ between E-A and E-I follicles. However, in E-I follicles, concentrations of i-IGF-I were positively correlated with progesterone levels ($r = 0.82$, $p < 0.01$) (Fig. 2B) and with numbers of LH binding sites in granulosa cells ($r = 0.28$, $p < 0.10$). Levels of i-IGF-I were negatively correlated with E_2 levels ($r = -0.45$), numbers of FSH binding sites in granulosa cells ($r = -0.54$) and numbers of LH binding sites in thecal cells ($r = -0.33$) (Table 3). Other correlations were not significant ($p > .10$, Table 3).

Experiment III.

Concentrations of i-IGF-I and progesterone in fluid of 1–4 mm, 5–12 mm or >12 mm follicles did not differ ($p > 0.10$) among size groups (Table 4). In contrast, E_2 was lower ($p < 0.05$) in 1–4 mm than in 5–12 mm or >12 mm follicles. Although follicles classified as E-A had 61-fold greater ($p < 0.01$) concentrations of E_2 , i-IGF-I levels were similar ($p > 0.10$) (Table 2). Progesterone levels were 2.6-fold greater in E-I than in E-A follicles ($p < 0.05$). Because of the low number of E-I follicles >4 mm ($n = 3$), correlation coefficients between i-IGF-I and E_2 or progesterone were not calculated. However, in E-A follicles ($n = 7$), concentrations of i-IGF-I were significantly correlated with diameter ($r = 0.72$, $p < 0.05$), and tended to correlate positively with progesterone levels ($r = 0.56$, $p < 0.10$) and inversely with E_2 levels ($r = -0.57$, $p < 0.10$).

DISCUSSION

Substantial evidence from in vitro experiments supports the notion that IGF-I at physiological concentrations (10–200 ng/ml) is stimulatory to granulosa cell steroidogenesis (Adashi et al., 1984; 1985a,b,c; Baranao and Hammond, 1984; Davoren et al., 1985; Hammond et al., 1985; Veldhuis et al.,

1985a,b; 1986a; 1987a,b; Schams, 1987; Hutchinson et al., 1988). However, it is more difficult to establish the importance of IGF-I in ovarian function in vivo. Thus, the present studies were undertaken to examine in detail the relationship between intrafollicular levels of IGF-I and various biochemical markers of follicular differentiation in individual follicles of cattle. Although we observed that concentrations of IGF-I in large (≥ 8 mm) follicles did not change with time postpartum or in response to LHRH injections, intrafollicular IGF-I levels were significantly correlated with several markers of follicular differentiation. The fact that concentrations of IGF-I were positively correlated with concentrations of progesterone in fluid from both E-A and E-I follicles of Experiments I, II, and/or III supports results of in vitro studies that have shown IGF-I as a potent stimulator (5- to 20-fold increase) of progesterone production by murine, bovine, and porcine granulosa cells (Adashi et al., 1984; 1985a,b,c,d; Baranao and Hammond, 1984; Veldhuis et al., 1985a; 1986a; 1987a,b; Davoren et al., 1986; Schams, 1987). In comparison, IGF-I is a much weaker (<5-fold increase) stimulator of E_2 production in cultured murine and porcine granulosa cells (Adashi et al., 1985a; Davoren et al., 1985; 1986; Veldhuis et al., 1985a), and this could explain, in part, why levels of E_2 and IGF-I in follicular fluid were not positively correlated in the present studies. In fact, levels of IGF-I and E_2 were negatively correlated in E-I follicles of Experiment II and in E-A follicles of Experiment III. This finding was surprising yet not without precedent, since Veldhuis et al. (1983) reported inhibitory effects of 1 μ g/ml insulin (a dose known to cross-react with the IGF-I receptor; Veldhuis et al., 1985b) on porcine granulosa cell aromatase in vitro. Although the current studies suggest that IGF-I is not rate-limiting for follicular E_2 production in cattle, they do not exclude the possibility that IGF-I acts as a permissive yet necessary factor for E_2 synthesis.

A significant positive correlation between follicular diameter and IGF-I levels was found in Experiments I and III, whereas only a weak positive correlation was observed in Experiment II of the present studies. These data are in partial agreement with those of Hammond et al. (1985) who reported that pooled fluid of large (>6 mm) porcine follicles contained 65% greater IGF-I levels than small (<3 mm) follicles. In addition, Hammond et al. (1988) reported that concentrations of i-IGF-I in follicular fluid (pooled within animal) increased coincident with an increase

in follicular size during preovulatory follicular development in gonadotropin-treated prepubertal gilts. Since these studies in swine evaluated a broader spectrum of follicular sizes than the present studies, perhaps a more consistent correlation would have been observed in E-A and E-I follicles across Experiments I and II if follicles <8 mm in diameter had been evaluated, as in Experiment III.

In both Experiments I and II, significant positive correlation between IGF-I levels and numbers of granulosa cell LH binding sites was observed. In agreement with our findings, IGF-I has been found to augment (by over 3-fold) FSH-induced increases in LH binding without affecting basal LH binding in cultured rat (Adashi et al., 1985d) and pig granulosa cells (Maruo et al., 1988). Although responsiveness of granulosa cells to FSH appears to be enhanced with addition of IGF-I (Adashi et al., 1985a,c; Davoren et al., 1985; 1986; Veldhuis et al., 1985a; 1987a; Maruo et al., 1988), this effect in rat granulosa cells is not accompanied by an increase in the number of FSH receptors (Adashi et al., 1988a). In comparison, we observed a significant negative correlation between IGF-I levels and numbers of granulosa cell FSH binding sites and thecal cell LH binding sites in E-I follicles in Experiment II. Further studies are needed to determine if numbers of thecal LH receptors are modulated by IGF-I.

The source of IGF-I in ovarian follicular fluid is uncertain. Since recent studies have shown that porcine granulosa cells can secrete immunoreactive IGF-I in vitro (Hammond et al., 1985; Hsu and Hammond, 1987a,b), local production of IGF-I within the follicle seems likely. However, IGF-I derived from serum and thecal cells must also be considered. It is also possible that changing follicular permeability to serum-derived IGF-I may modulate concentrations of IGF-I in follicular fluid. These possibilities await further elucidation. Regardless of the source of follicular fluid IGF-I, the concentrations measured in follicular fluid of the present studies are within the range of concentrations that have been shown to be effective in stimulating steroidogenesis (Schams, 1987) and proliferation (Savion et al., 1981) of bovine granulosa cells in vitro.

In summary, dramatic increases in concentrations of E_2 in follicular fluid were not associated with increases in levels of IGF-I during spontaneous and LHRH-induced follicular development in cattle. Thus, these data do not support the hypothesis that IGF-I

levels in follicular fluid enhance estrogen biosynthesis in vivo, although a permissive role for IGF-I can not be excluded. In fact, two of three studies revealed an inverse relationship between IGF-I and E_2 . On the other hand, increased concentrations of progesterone in follicular fluid were associated with increased levels of IGF-I, and thus support the hypothesis that IGF-I levels in follicular fluid regulate progesterone biosynthesis in vivo.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. K. Leung and S. Lyth for technical assistance; Drs. J. J. Van Wyk and L. E. Underwood for providing the IGF-I antiserum through the National Hormone and Pituitary Program; Dr. M. R. Sairam (Montreal, Quebec, Canada) for supplying highly purified ovine FSH; Dr. G. D. Niswender (Colorado State University) for supplying antiserum to androstenedione; the National Hormone and Pituitary Program for supplying bovine LH and hCG (Cr-119); and Mrs. Sandra J. Christian for her excellent secretarial assistance.

REFERENCES

- Adashi EY, Resnick CE, Brodie AMH, Svoboda ME, Van Wyk JJ, 1985a. Somatomedin-C-mediated potentiation of follicle-stimulating hormone-induced aromatase activity of cultured rat granulosa cells. *Endocrinology* 117:2313-20
- Adashi EY, Resnick CE, D'Ercole AJ, Svoboda ME, Van Wyk JJ, 1985b. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocr Rev* 6:400-20
- Adashi EY, Resnick CE, Hernandez ER, May JV, Knecht M, Svoboda ME, Van Wyk JJ, 1988a. Insulin-like growth factor-I as an amplifier of follicle-stimulating hormone action: studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. *Endocrinology* 122:1583-91
- Adashi EY, Resnick CE, Hernandez ER, Svoboda ME, Van Wyk JJ, 1988b. Characterization and regulation of a specific cell membrane receptor for somatomedin-C/insulin-like growth factor I in cultured rat granulosa cells. *Endocrinology* 122:194-201
- Adashi EY, Resnick CE, Svoboda ME, Van Wyk JJ, 1984. A novel role for somatomedin-C in the cytodifferentiation of the ovarian granulosa cell. *Endocrinology* 115:1227-29
- Adashi EY, Resnick CE, Svoboda ME, Van Wyk JJ, 1985c. Somatomedin C synergizes with follicle-stimulating hormone in the acquisition of progestin biosynthetic capacity by cultured rat granulosa cells. *Endocrinology* 116:2135-42
- Adashi EY, Resnick CE, Svoboda ME, Van Wyk JJ, 1985d. Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* 116:2369-75
- Adashi EY, Resnick CE, Svoboda ME, Van Wyk JJ, 1986. Follicle-stimulating hormone enhances somatomedin-C binding to cultured rat granulosa cells (evidence for cAMP dependence). *J Biol Chem* 261:3923-26
- Baranao JLS, Hammond JM, 1984. Comparative effects of insulin and insulin-like growth factors on DNA synthesis and differentiation of porcine granulosa cells. *Biochem Biophys Res Commun* 124:484-90
- Davoren JB, Hsueh AJW, Li CH, 1985. Somatomedin C augments FSH-induced differentiation of cultured rat granulosa cells. *Am J Physiol* 249:E26-33
- Davoren JB, Kasson BG, Li CH, Hsueh AJW, 1986. Specific insulin-like growth factor (IGF)-I- and II-binding sites on rat granulosa cells: relation to IGF action. *Endocrinology* 119:2155-62
- Furlanetto RW, Marino JM, 1987. Radioimmunoassay of somatomedin C/insulin-like growth factor I. *Methods Enzymol* 146:216-26

- Hammond JM, Baranao JLS, Skaleris D, Knight AB, Romanus JA, Rechler MM, 1985. Production of insulin-like growth factors by ovarian granulosa cells. *Endocrinology* 117:2553-55
- Hammond JM, Hsu C-J, Klindt J, Tsang BK, Downey BR, 1988. Gonadotropins increase concentrations of immunoreactive insulin-like growth factor-I in porcine follicular fluid *in vivo*. *Biol Reprod* 38:304-08
- Hsu C-J, Hammond JM, 1987a. Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells *in vitro*. *Endocrinology* 120:198-207
- Hsu C-J, Hammond JM, 1987b. Concomitant effects of growth hormone on secretion of insulin-like growth factor-I and progesterone by cultured porcine granulosa cells. *Endocrinology* 121:1343-48
- Hutchinson LA, Findlay J, Herington AC, 1988. Growth hormone and insulin-like growth factor-I accelerate PMSG-induced differentiation of granulosa cells. *Mol Cell Endocrinol* 55:61-69
- Ireland JJ, Roche JF, 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid and gonadotropin receptors. *Endocrinology* 111:2077-86
- Maruo T, Hayashi M, Matsuo H, Ueda Y, Morikawa H, Mochizuki M, 1988. Comparison of the facilitative roles of insulin and insulin-like growth factor I in the functional differentiation of granulosa cells: *in vitro* studies with the porcine model. *Acta Endocrinol* 117:230-40
- Ott L, 1977. *An Introduction to Statistical Methods and Data Analysis*. North Scituate, MA: Duxbury Press, pp. 384-86
- Savion N, Lui GM, Laherty R, Gospodarowicz D, 1981. Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* 109:409-20
- Schams D, 1987. Luteal peptides and intracellular communication. *J Reprod Fertil* 34:87-99 (Suppl.)
- Spicer LJ, Convey EM, Leung K, Short RE, Tucker HA, 1986a. Anovulation in postpartum suckled beef cows. II. Associations among binding of ¹²⁵I-labeled gonadotropins to granulosa and thecal cells, and concentrations of steroids in serum and various sized ovarian follicles. *J Anim Sci* 62:742-50
- Spicer LJ, Convey EM, Tucker HA, Echterkamp SE, 1986b. Effects of intermittent injections of LHRH on specific binding of ¹²⁵I-labeled gonadotropins to granulosa and theca, and concentrations of steroids in serum and ovarian follicles during postpartum anovulation in suckled beef cows. *J Anim Sci* 62:1324-31
- Spicer LJ, Convey EM, Tucker HA, Echterkamp SE, 1986c. Effects of intermittent injections of LHRH on secretory patterns of LH and FSH and ovarian follicular growth during postpartum anovulation in suckled beef cows. *J Anim Sci* 62:1317-23
- Spicer LJ, Ireland JJ, 1986. Specific binding of ¹²⁵I-labeled human chorionic gonadotropin to gonadal tissue: comparison of limited-point saturation analyses to Scatchard analyses for determining binding capacities and factors affecting estimates of binding capacity. *Anal Biochem* 156:25-30
- Spicer LJ, Leung K, Convey EM, Gunther J, Short RE, Tucker HA, 1986d. Anovulation in postpartum suckled beef cows. I. Associations among size and numbers of ovarian follicles, uterine involution, and hormones in serum and follicular fluid. *J Anim Sci* 62:734-41
- Veldhuis JD, Demers LM, Garmey J, Juchter D, Azimi P, 1985a. A role of somatomedin C as a differentiating hormone and amplifier of hormone action on ovarian cells: studies with synthetically pure human somatomedin C and swine granulosa cells. *Biochem Biophys Res Commun* 130:234-40
- Veldhuis JD, Furlanetto RW, Juchter D, Garmey J, Veldhuis P, 1985b. Trophic actions of human somatomedin C/insulin-like growth factor I on ovarian cells: *in vitro* studies with swine granulosa cells. *Endocrinology* 116:1235-42
- Veldhuis JD, Kolp LA, Toaff ME, Strauss JF, Demers LM, 1983. Mechanisms subserving the trophic actions of insulin on ovarian cells: *in vitro* studies using swine granulosa cells. *J Clin Invest* 72:1046-57
- Veldhuis JD, Nestler JE, Strauss JF, Azimi P, Garmey J, Juchter D, 1987a. The insulin-like growth factor, somatomedin-C, modulates low density lipoprotein metabolism by swine granulosa cells. *Endocrinology* 121:340-46
- Veldhuis JD, Rodgers RJ, Azimi P, Garmey J, Juchter D, May W, 1987b. Mechanisms subserving the steroidogenic synergism between follicle-stimulating hormone and insulin-like growth factor-I (somatomedin C): alterations in cellular sterol metabolism in swine granulosa cells. *J Biol Chem* 262:7658-64
- Veldhuis JD, Rodgers RJ, Dee A, Simpson ER, 1986a. The insulin-like growth factor, somatomedin C, induces the synthesis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in ovarian cells. *J Biol Chem* 261:2499-2502
- Veldhuis JD, Rodgers RJ, Furlanetto RW, Azimi P, Juchter D, Garmey J, 1986b. Synergistic actions of estradiol and the insulin-like growth factor somatomedin-C on swine ovarian (granulosa) cells. *Endocrinology* 119:530-38