Synergistic Effects of Insulin-Like Growth Factor-I and Human Chorionic Gonadotropin in the Rat Ovary

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Insulin and low doses of luteinizing hormone (LH) activity (human chorionic gonadotropin [hCG]) act synergistically in the rat to produce anovulation, large ovarian cysts, and elevated plasma androstenedione levels. Further, both insulin and insulin-like growth factor-I (IGF-I) affect the ability of gonadotropins to enhance both ovarian theca and granulosa cell function in vitro. The present series of experiments were performed to determine if recombinant human IGF-I (rhIGF-I) can act in a manner similar to insulin when combined with subovulatory doses of hCG in adult normally cycling rats. Fifty-four female Sprague-Dawley rats were randomly assigned to the following treatment groups at the age of 64 days: (A) vehicle alone (controls, phosphate-buffered saline containing 0.09% pig gelatin), (B) twice-daily subcutaneous injections of 0.5 to 3.0 U insulin, (C) twice-daily subcutaneous injections of 1.5 U hCG, (D) both insulin and hCG, (E) twice-daily subcutaneous injections of rhIGF-I (2.5 mg/kg/d), and (F) both hCG and rhIGF-I. After 22 days of treatment, the animals were killed on day 23, trunk blood was collected, and the ovaries were excised for histological study. Eight of 9 control rats and 5 or 6 of 9 rats treated with insulin, hCG, or rhIGF-I alone displayed normal estrus cycles throughout the in vivo treatment period as assessed by daily vaginal smears. In marked contrast, only 1 animal treated with hCG + insulin and 2 animals treated with hCG + rhIGF-I continued to display vaginal smears indicative of normal cycling. Multiple large ovarian follicular cysts were found only in these latter 2 groups (3 of 9 animals in each group). Mean serum testosterone levels were significantly elevated in animals receiving insulin + hCG (0.72 ± 0.28 *v* 0.17 ± 0.03 ng/mL in controls, $P = .05$). Mean serum androstenedione levels were significantly elevated in animals receiving hCG and animals receiving rhIGF-I + hCG (5.57 ± 0.99 and 2.39 ± 0.68 ng/mL, respectively, *v* 0.14 ±0.14 ng/mL in controls, $P < .01$ and $P < .05$, respectively). We conclude that rhIGF-I and insulin act synergistically with subovulatory doses of hCG to disrupt normal reproductive cycling, elevate serum androgen concentrations, and induce large ovarian cysts in intact adult rats.

A NUMBER OF in vitro and in vivo studies have demonstrated a potential physiological role for insulin as a modulator of gonadotropic action at the level of ovarian follicular compartments.[1] Insulin, for example, has been shown to increase the secretion of progesterone in cultured porcine granulosa cells,[2,3] to increase the secretion of both progesterone and androstenedione in cultured porcine thecal cells,[4] and to augment the effects of gonadotropins on ovarian cellular steroidogenesis.[2,3,5,6] In rats, experimental hyperinsulinemia produced downregulation of ovarian insulin receptors and upregulation of ovarian insulin-like growth factor-I (IGF-I) receptors.[7] Experimental hyperinsulinemia also has been shown to act synergistically with low doses of luteinizing hormone (LH)-like activity (human chorionic gonadotropin [hCG]) to induce bilateral large ovarian follicular cysts and increase plasma androstenedione levels in the rat.[8] There is a growing body of in vitro evidence that IGF-I also may act synergistically with gonadotropins to affect ovarian function. IGF-I appears to act synergistically with gonadotropins in rat granulosa cells with regard to progesterone production,[9,10] aromatase activity,[10] and LH receptor synthesis.[11] In addition, IGF-I augments LH-induced androgen biosynthesis in rat theca cells.[12,13]

Recombinant human IGF-I (rhIGF-I) has recently become available for experimental use. Its effects have included growth restoration in both insulin-deficient diabetic rats and hypophysectomized rats,[14,15] as well as increased organ size, cancellous bone deposition, and lymphopoiesis.[16-18] The present series of experiments were undertaken to determine whether rhIGF-I can act synergistically with subovulatory doses of LH-like activity (hCG) in regulating rat ovarian function.

MATERIALS AND METHODS

Experimental Animals

The following in vivo procedures were performed in the Cornell University Medical College Animal Facility according to a protocol approved by the Institutional Animal Care and Use Committee. Sixty-five female Sprague-Dawley rats (180 to 200 g; Hilltop Laboratories, Scottsdale, PA) were housed 5 per cage at 42 days of age, fed standard rat chow, given water ad libitum, and kept in a room of constant humidity and temperature with controlled lighting (12 hours light:12 hours dark). The animals underwent a 21-day period of initial monitoring that included daily vaginal smears to document consecutive 4- to 5-day estrus cycles. Only animals that demonstrated normal cycling were entered into the treatment phase of the experiment. All rats were given free access to rat chow, 5% dextrose in water, and sugar cubes. All animals continued to undergo daily vaginal smears and were weighed twice weekly. They were considered to be cycling if they completed at least 1 estrus cycle during each 7-day period.

Experimental Design

Injections were performed twice daily via subcutaneous administration in the scruff of the neck in a vol of 0.2 mL. Vehicle (phosphate-buffered saline containing 0.09% pigskin gelatin, Sigma Chemical, St Louis, MO; pH 7.0) was used for excipient injections. Insulin (NPH-Humulin U-100; Lilly, Indianapolis, IN) was administered as previously described to produce a hyperinsulinemic state without hypoglycemia.[8] hCG (Steris Laboratories, Phoenix, AZ) was administered at a dose that has been previously shown to induce ovarian follicular cysts in both progesterone-synchronized immature rats and intact mature rats.[8,19] This dose of hCG also does not exceed the threshold expected to induce

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Submitted March 26, 1999; accepted August 16, 1999.

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0026-0495-00/4903-0006$10.00/0

ovulation.[20] rhIGF-I (Chiron, Emeryville, CA) was administered at a dose known to have identifiable physiologic effects such as increased body/organ weights in rats.[21,22] The experimental animals were randomized into the following 6 weight-matched treatment groups: (A) vehicle, twice-daily subcutaneous injections, n = 9; (B) insulin, twice-daily subcutaneous injections, 0.5 to 6.0 U · d, n = 9; (C) hCG, twice-daily subcutaneous injections, 3.0 IU · d, n = 9; (D) insulin and hCG, twice-daily subcutaneous injections, n = 9; (E) rhIGF-I, twice-daily subcutaneous injections, 2.5 mg/kg · d, n = 9; and (F) rhIGF-I and hCG, twice-daily subcutaneous injections, n = 9 (Table 1).

Tissue Harvest

The animals were decapitated 12 to 16 hours after the last injection on day 23 of treatment. Trunk blood was collected in polypropylene tubes for measurement of serum hormone levels. The ovaries were excised, adhering tissue was removed, and the ovaries were weighed and immediately fixed in Bouin's solution.

Histology

Ovarian sections were prepared and stained with hematoxylin-eosin by the Histology Core Facility at the University of South Carolina School of Medicine. An ocular micrometer on a Zeiss 35IM microscope (Carl Zeiss, Oberkochen, Germany) was used to obtain follicular and ovarian measurements. The mean ovarian area was obtained from histologic analysis of sections that represented the maximal cross-sectional area for each ovary. Ovaries were classified as cystic when they contained multiple follicular cysts greater than 0.8 mm in diameter with stimulated thecal tissue and just a remnant of granulosa cells, as previously described.[8] Photomicrographs were obtained with a 2.5× objective.

Radioimmunoassays

Blood samples were allowed to clot on ice and were then centrifuged at 3,000 × *g* for 7 minutes. The resultant serum samples were transferred to individual polypropylene tubes, frozen, and shipped on dry ice to the University of South Carolina School of Medicine (K.B.). These samples were maintained frozen at −90°C until analysis for IGF-I, hCG, and insulin content. Radioimmunoassay (RIA) kits for rat IGF-I, human IGF-I, hCG, and insulin were obtained from Diagnostic Systems Laboratories ([DSL] Webster, TX).

Serum steroid content was determined using established sample-extraction, high-performance liquid chromatography (HPLC), and RIA protocols as previously described.[23] Briefly, each sample was extracted individually with HPLC-grade diethyl ether (Fisher Scientific, Pittsburgh, PA) in the presence of the authentic tritiated form of each steroid of interest (1,500 cpm; Dupont New England Nuclear, Boston, MA) to monitor procedural losses. The ether extract residues were reconstituted in acetonitrile ([AcN] Fisher Scientific), which was brought to a final AcN:H_2O ratio of 40:60 with filtered MilliQ-grade water (Millipore Filter, Bedford, MA) and chromatographed. Samples were collected and reconstituted in assay buffer as previously described.[23] Antisera against androstenedione and testosterone were generously provided by Drs Gerry Nordblom and Barry England (Ann Arbor, MI). Antisera against estrone were obtained from DSL. The iodinated forms of androstenedione, estradiol, and estrone were obtained from DSL, and the iodinated form of testosterone was obtained from Diagnostic Products (Los Angeles, CA). Steroid RIAs were performed as previously described.[19,23,24]

Table 1. Schedule of Insulin Injections

Day	Insulin Dose (U/d)
1	0.5
2	1.5
3	1.5
4	2.0
5	2.5
6	3.0
7	4.0
8	4.0
9	5.0
10	5.0
11-22	6.0

Table 2. Effects of Insulin, hCG, and rhIGF-I on Body Weight

Group	Initial Weight (g)	Final Weight (g)	Weight Change (g)
Vehicle	229 ± 4	259 ± 7	30 ± 4
Insulin	228 ± 4	272 ± 6	44 ± 4*
hCG	232 ± 4	258 ± 8	26 ± 4
Insulin + hCG	228 ± 4	271 ± 6	41 ± 2
rhIGF-I	238 ± 5	296 ± 6	58 ± 3†
rhIGF-I + hCG	230 ± 4	273 ± 5	45 ± 4*

*$P < .05$, †$P < .01$ *v* vehicle, 1-way ANOVA and 2-sided Dunnett's test.

Statistics

Continuous data were analyzed by 1-way ANOVA with a 2-sided Dunnett's test for comparisons between the experimental treatment groups and controls. Proportions were analyzed by Fisher's exact test. A *P* value of .05 or less was considered statistically significant. All data are represented as the mean ± SE of 9 animals per group.

RESULTS

Animal Weight

All animals gained weight during the in vivo treatment period (Table 2). However, animals treated with insulin ($P < .05$), rhIGF-I ($P < .01$), or rhIGF-I + hCG ($P < .05$) gained significantly more weight than control rats. Neither hCG alone nor insulin + hCG produced weight gains significantly larger than those found in control animals.

Reproductive Cycle

The fraction of cycling animals in each treatment group was calculated for 3 7-day periods during the in vivo treatment phase (Table 3). All animals treated with vehicle alone continued to cycle normally, except for 1 animal that lost reproductive cyclicity during the final week of the treatment period. Five or 6 of 9 animals in the treatment groups on either insulin, hCG, or rhIGF-I continued to exhibit normal cycles during the latter 2 weeks of the in vivo treatment period. In contrast, during the second treatment week, only 1 animal in the insulin + hCG treatment group and no animals in the rhIGF-I + hCG treatment group cycled normally ($P < .01$). Two animals in the rhIGF-I + hCG treatment group that did not cycle in the second week resumed cycling in the third week of the treatment phase.

Ovarian Morphology

Ovarian morphologic parameters are presented in Table 4. No statistically significant differences were observed in ovarian weight for the hormonally treated groups compared with the control group. However, significant increases in the mean maximal ovarian cross-sectional area were observed for the

Table 3. Fraction of Animals With Continued Normal Reproductive Cycling

Group	Days 1-7	Days 8-14	Days 15-21
Vehicle	9/9	9/9	8/9
Insulin	8/9	6/9	5/9
hCG	8/9	5/9	6/9
Insulin + hCG	9/9	1/9†	1/9†
rhIGF-I	7/9	5/9	6/9
rhIGF-I + hCG	9/9	0/9†	2/9*

NOTE. Animals were considered to be cycling if they completed at least 1 estrous cycle during the designated periods.

*$P < .05$, †$P < .01$ *v* vehicle, Fisher's exact test.

rhIGF-I + hCG ($P < .01$) treatment group compared with controls. There were no instances of ovaries with cystic follicles in the vehicle, insulin, hCG, or rhIGF-I treatment groups. However, bilateral cystic ovaries were found in 3 of 9 cases, for both the insulin + hCG and rhIGF-I + hCG treatment groups (Fig 1).

Serum Peptide and Hormone Concentrations

Serum peptide and steroid hormone concentrations are depicted in Fig 2. Serum insulin concentrations were 24.9 ± 5.8 to 30.87 ± 5.7 mIU/mL in groups that received insulin injections. These values were significantly greater than the levels observed for rats that received vehicle (9.2 ± 0.7 mIU/mL, $P < .01$). Serum hCG concentrations were undetectable in nearly all animals that received hCG injections (9 of 9 hCG-treated animals, 5 of 9 insulin + hCG–treated animals, and 5 of 9 rhIGF-I + hCG–treated animals). When detected, hCG concentrations were invariably just above the threshold of detection for the RIA used (4 mIU/mL).

Human IGF-I concentrations were detected only in animals that received rhIGF-I (108.3 ± 16.1 to 113.8 ± 15.7 ng/mL). Rat IGF-I levels were significantly lower in animals treated with insulin + hCG (1,262.0 ± 116.0 ng/mL) or rhIGF-I + hCG (1,201 ± 179.0 ng/mL) compared with controls (1,747.7 ± 113.1 ng/mL, $P < .05$).

Serum testosterone concentrations were significantly elevated in the insulin + hCG group (0.72 ± 0.28 ng/mL) compared with the controls (0.17 ± 0.03 ng/mL, $P = .05$). Serum androstenedione levels were significantly elevated in the hCG (5.57 ± 0.99 ng/mL, $P < .01$) and rhIGF-I + hCG (2.39 ± 0.68 ng/mL) groups in comparison to the controls (0.14 ± 0.14 ng/mL, $P < .05$). Serum estradiol and estrone levels were not significantly different from the control levels in any treatment group.

Table 4. Ovarian Morphologic Parameters

Group	Weight (mg)	Area (mm^2)	Bilaterally Cystic Ovaries (%)
Vehicle	48 ± 2	11.4 ± 0.7	0.0
Insulin	38 ± 3	11.1 ± 0.9	0.0
hCG	37 ± 2	12.0 ± 0.8	0.0
Insulin + hCG	46 ± 2	14.5 ± 0.7	33.0
rhIGF-I	42 ± 6	14.5 ± 1.0	0.0
rhIGF-I + hCG	54 ± 6	17.9 ± 1.5*	33.0

NOTE. Area determinations were made through histologic analysis of sections that represent maximal cross-sectional area measurements for each ovary.

*$P < .01$ *v* vehicle, 1-way ANOVA and 2-sided Dunnett's test.

DISCUSSION

Ovarian follicular cysts develop in response to unabated stimulation by subovulatory doses of hCG in both progesterone-synchronized immature rats and pregnant rats.[19,24] In contrast, ovarian cysts do not develop spontaneously in adult hyperinsulinemic rats.[7] However, insulin acts synergistically with low-dose hCG in the adult cycling rat to increase the size of follicular cysts as well as the incidence of large bilateral ovarian cysts.[8] Further, tonic stimulation by follicle-stimulating hormone (FSH) plus growth hormone (GH) results in the induction of ovarian cysts in hypophysectomized rats.[25]

Studies of the potential effects of insulin and IGF-I at the level of the ovary have centered mostly on the effects of these hormones on the steroidogenic ability and capacity of the individual follicular compartments in vitro.[26,27] It has been more difficult to demonstrate an in vivo effect of hyperinsulinemia on ovarian steroid secretion (eg, euglycemic-hyperinsulinemic clamp studies in the human).[28-30]

Many investigators have hypothesized that insulin may affect ovarian function and the development of ovarian cysts in insulin-resistant/hyperinsulinemic individuals by acting through the type 1 IGF receptor which is present on both human and rodent granulosa and thecal cells.[31] Although insulin can bind to this receptor, its affinity for the type 1 IGF receptor is much less than that of IGF-I.[32,33] Therefore, under normal physiologic conditions, insulin would not be expected to have a significant effect on the ovary through the type 1 IGF receptor. In support of this concept, recent studies using monoclonal antibodies against the IGF-I receptor clearly demonstrate that insulin acts on the ovary primarily through its own receptor.[34] Thus, it has been suggested that the retention of insulin's effects on ovarian function in certain insulin-resistant patients may be due to the specific lack of down regulation of the insulin receptor in premenopausal females[35] and/or preservation of postreceptor signaling events other than those involved in glucose transport.[31,36]

Experimental hyperinsulinemia in the rat upregulates ovarian IGF-I binding sites.[7] In addition, hyperinsulinemia suppresses IGF-binding protein-1 (IGFBP-1) production both in the liver and in the ovary.[37-40] Further, IGF-I produced by granulosa cells in response to stimulation by GH may enhance the ability of rat thecal cells to produce androgens in response to stimulation by LH.[12,13] GH, which stimulates production of IGF-I, promotes FSH-induced induction of ovarian cysts in hypophysectomized rats.[25] Therefore, we proposed that IGF-I might act synergistically with subovulatory doses of LH-like activity to produce anovulation, ovarian stromal and thecal hyperplasia, and cyst formation in otherwise intact, cycling rats. We used rhIGF-I to test this hypothesis.

Both insulin and IGF-I displayed synergistic effects with hCG in this study. First, only animals that received combined treatment with either insulin + hCG or rhIGF-I + hCG displayed large ovarian cysts by the end of the in vivo treatment regimen. Second, nearly all animals in the combined treatment groups stopped cycling by the end of the in vivo treatment regimen, whereas less than half of the animals treated with

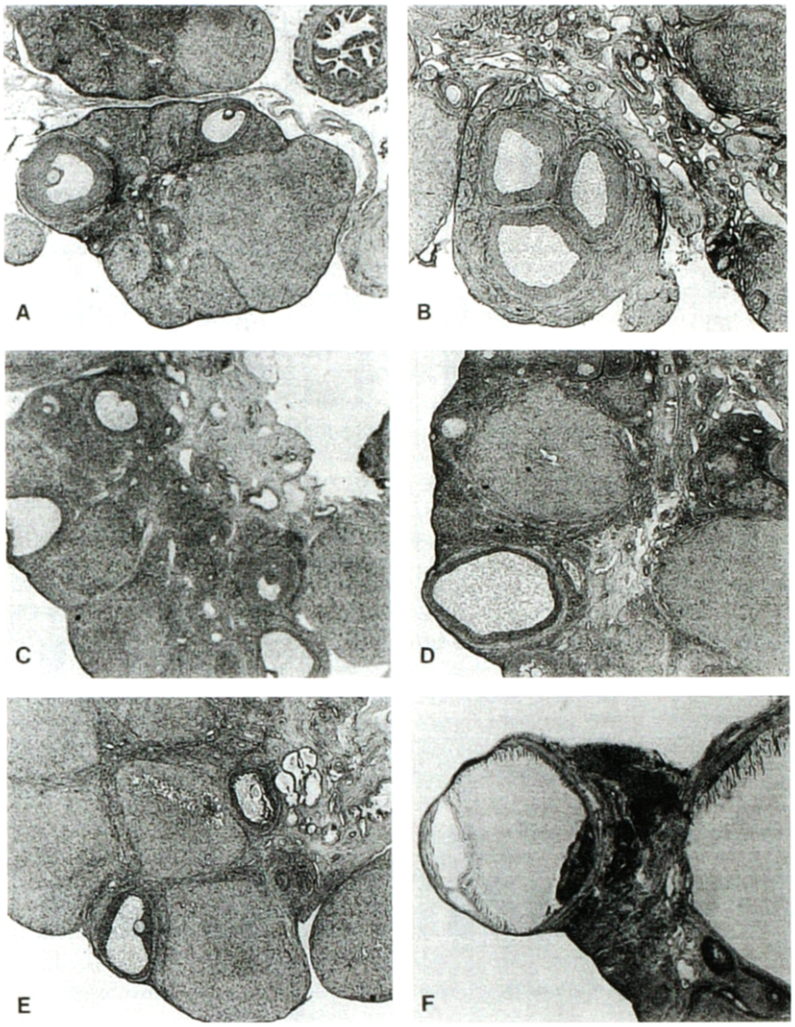

Fig 1. Histological sections from animals in the in vivo hormonal treatment groups: (A) vehicle alone, (B) insulin, (C) hCG, (D) insulin + hCG, (E) rhIGF-I, (F) rhIGF-I + hCG. Photomicrographs were made with a 2.5× objective.

insulin, rhIGF-I, or hCG alone stopped cycling. Third, the mean ovarian cross-sectional area was larger for animals treated with rhIGF-I + hCG versus control rats.

In contrast to the synergistic effects of insulin + hCG or rhIGF-I + hCG on gross ovarian appearance, only combined treatment with insulin + hCG resulted in a synergistic increase in serum testosterone compared with control values by the end of the in vivo treatments. In contrast, serum androstenedione concentrations increased in response to hCG alone. It is tempting to speculate from these observations that the heterogenous patterns of androgen elevation in women with polycystic ovarian syndrome (PCOS) may be due, in part, to the degree to which insulin resistance, hyperinsulinemia, IGF-I, or all 3 factors may be involved.

It is not surprising that serum estrogen concentrations were not elevated in the cyst-bearing animals by the end of the in vivo treatment period. It has been shown previously in hypophysectomized rats that serum estradiol increases initially in response to combined FSH + hCG treatment but decreases dramatically once the follicles begin to lose granulosa cells and assume a cystic appearance.[23] A similar effect may have occurred in the present model.

We noted that endogenous rat IGF-I serum levels were significantly lower in animals receiving insulin + hCG and rhIGF-I + hCG in comparison to controls. Lower rat IGF-I levels in animals receiving insulin may occur through inhibition of certain hepatic IGFBPs, similar to the known effects of insulin on hepatic IGFBP-1 production in humans.[37,38] However, it is unclear as to why rat IGF-I levels were significantly lower in animals receiving insulin + hCG and not also in animals receiving insulin alone. Lower rat IGF-I levels in animals receiving exogenous rhIGF-I may be associated with the suppression of either GH or IGFBPs that are GH-dependent such as IGFBP-3 in humans.[41] However, it is unclear why rat IGF-I levels were significantly lower only in animals receiving rhIGF-I + hCG and not also in animals receiving rhIGF-I alone.

The majority of animals receiving hCG had unmeasurable hCG concentrations at the time of death (12 to 16 hours after the last hCG injection). When hCG concentrations were detectable, the levels were just above the threshold of detection for the RIA (4 mIU/mL). This occurred invariably as a result of the use of very small dosages of hCG (1.5 IU twice daily) coupled with the fact that hCG has a very short half-life when administered exogenously in the rat.[42,43] The hCG results therefore reveal an

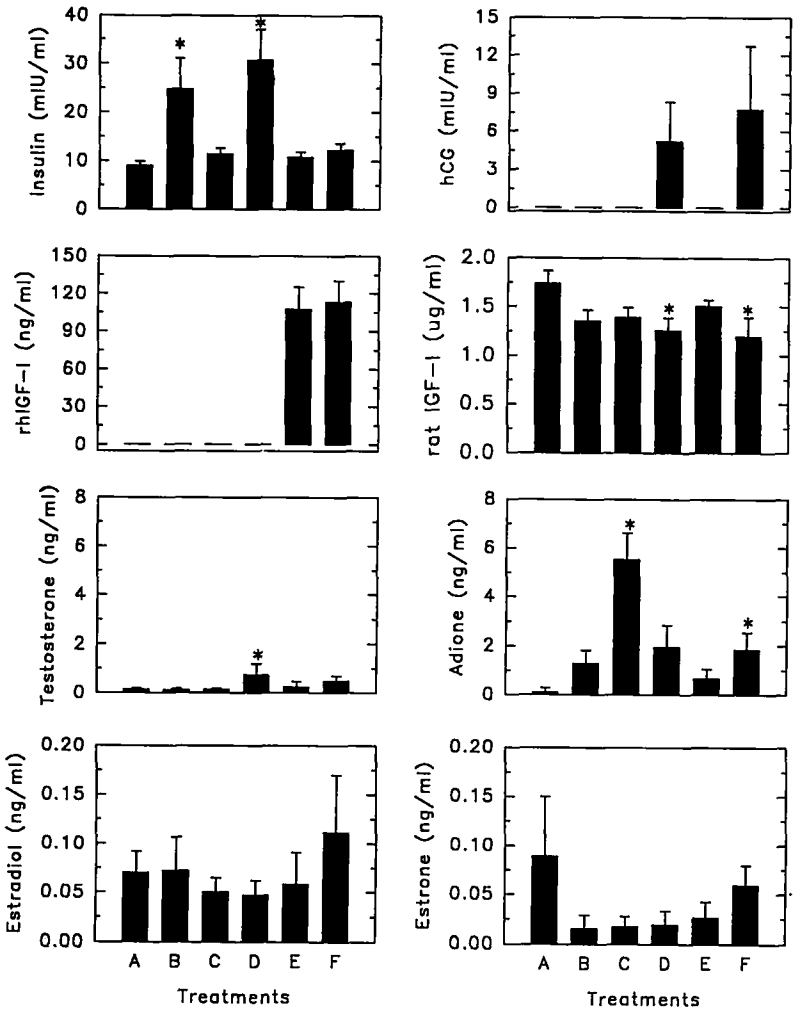

Fig 2. Serum hormone concentrations in response to the in vivo hormonal treatments. Each bar represents the mean ± SE for sera from 9 rats. X-axis: (A) vehicle alone, (B) insulin, (C) hCG, (D) insulin + hCG, (E) rhIGF-I, (F) rhIGF-I + hCG. *$P \leq .05$ *v* vehicle, 1-way ANOVA and 2-sided Dunnett's test.

ascertainment limitation of our study design rather than any appreciable between-group differences (all hCG-treated animals received identical dosages).

Although the data presented here reveal intriguing information regarding the potential role for IGFs in ovarian cyst development in the rat, our ability to correlate these data directly with the situation in humans is limited at this time. Indeed, IGF-II appears to be the major IGF expressed in the human ovary.[44,45] Still, even if the true endogenous IGF in the human ovary is IGF-II, the effects of this growth factor on thecal function may occur primarily through the type 1 IGF receptor, since the type 2 IGF/mannose-6-phosphate receptor appears to be largely metabolically inactive.[46] On the other hand, although appreciable between-species differences in the expression of IGF ligands have been noted, type 1 IGF receptor gene expression is nearly identical in both the rat and the human ovary.[47] One might therefore expect similar ovarian actions in response to varying systemic IGF-I concentrations.

It is important to note that while there are some similarities with the human PCOS condition noted in this animal model, there are dissimilarities as well. In the rat model, ovarian cysts are commonly large and relatively acellular. This differs from the ovarian morphology most typically found in human PCOS in which the ovaries demonstrate multiple small antral follicular cysts that show arrested development.

In summary, both insulin and IGF-I appear to act synergistically with low doses of hCG (LH-like activity) to induce anovulation, hyperandrogenism, and the development of ovarian follicular cysts in rats. This animal model demonstrates that IGF-I has systemic ("hormonal") effects on ovarian physiology in addition to its suspected local effects. In addition, this animal model may be useful in further studies exploring factors associated with ovarian cyst formation, as well as the pathogenesis of PCOS in humans.

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