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Comparison of Growth Hormone and Insulin-Like Growth Factor-I Regulation of Estradiol and Progesterone Production in Human Luteinized Granulosa Cells

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ABSTRACT

Growth hormone (GH) appears to affect the timing of puberty in children. The effects of GH on puberty may be related to direct GH action on ovarian function or may be mediated by IGF-I. To determine the likelihood that GH has direct effects on ovarian function, we compared the ability of GH and IGF-I to increase luteinized granulosa cell steroidogenesis in the absence and presence of gonadotropins. Cells were obtained from women undergoing *in vitro* fertilization for tubal disorders or male factor infertility and were placed in static culture. GH alone failed to alter progesterone or estradiol accumulation in the medium of cultured luteinized granulosa cells. IGF-I produced no increase in progesterone accumulation but increased estradiol accumulation 5.6-fold compared with cells treated with vehicle. The combination of GH and FSH produced an 0.83-fold increase in estradiol accumulation, whereas the combination of IGF-I and FSH resulted in a 2.9-fold increase in estradiol accumulation above FSH alone. Thus the direct effects of GH on granulosa cell steroid synthesis are modest compared with those of IGF-I. If GH has an effect on ovarian development at puberty, it is likely to be mediated by a GH-induced increase in circulating IGF-I. (***Pediatr Res* 38: 763–767, 1995**)

Abbreviations

GH, growth hormone
IGFBP-3, insulin-like growth factor binding protein-3

GH is thought to play a role in the timing of the onset of puberty. GH-deficient children have delayed adolescent development, and the timing of their puberty can be accelerated by administration of exogenous GH (1). Exogenous GH has also been found to augment the ability of administered FSH to induce follicular development in women with hypopituitarism and in women undergoing *in vitro* fertilization procedures (2–5). The ability of GH to augment gonadotropin action in the ovary may be related to an increase in IGF-I which is produced in response to exogenous GH (6). IGF-I has been shown to augment gonadotropin stimulation of steroidogenesis in human granulosa cells (7). GH, however, may be able to stimulate ovarian development or steroidogenesis directly, because GH receptors have been demonstrated in the human ovary (8), and, in animal studies, GH treatment increases steroidogenesis and gonadotropin receptor development (9, 10).

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To better understand how GH may affect ovarian development, we compared the ability of GH and IGF-I to increase steroidogenesis in luteinized human granulosa cells obtained from women undergoing *in vitro* fertilization procedures. We tested the hypothesis that GH, like IGF-I, would directly stimulate estradiol and progesterone production in luteinized granulosa cells maintained in static culture.

METHODS

Subjects. Luteinized ovarian granulosa cells were obtained during oocyte retrieval from women undergoing *in vitro* fertilization procedures for either tubal disorders or male factor infertility. Oral consent for use of the cells was obtained from each woman, and the consent procedure and protocols were approved by the Institutional Review Board of the University of Michigan. All received treatment with a gonadotropin-releasing hormone agonist for suppression of endogenous gonadotropins, human menopausal gonadotropins, and human chorionic gonadotropin administered 35 h before oocyte retrieval.

Materials. Medium preparations, antibiotics, L-glutamine, and FCS were purchased from either Irvine (Santa Ana, CA) or

Life Technologies, Inc., Grand Island, NY). Epidermal growth factor, thyroxin, hydrocortisone, transferrin, retinol acetate, methylisobutylxanthine, and androstenedione were purchased from Sigma Chemical Co. (St. Louis, MO). BSA (CRG-7) was from Armor (Kankakee, IL). Human recombinant-DNA-derived 22,000-D GH and human recombinant DNA-derived-IGF-I were a gift from Lilly Research Laboratories (Indianapolis, IN). LH (WHO 78-549) and FSH (I-3) were obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Rockville, MD).

Cell culture. Cells received after oocyte retrieval were centrifuged from follicular fluid at 100 × *g*. The cells were suspended in Ham's F-10 medium, layered over Ficoll-Hypaque (Pharmacia Biotech Inc., Piscataway, NJ), and centrifuged for 15 min at 100 × *g*. The layer at the medium-Ficoll interface was removed, washed three times in Ham's F-10 medium, and suspended in Ham's F-10 medium containing 10% FCS and 20 ng/mL gentamicin except as noted. The number of granulosa cells present was estimated using a hemocytometer, and greater than 95% of the cells were viable by trypan blue dye exclusion. As an additional test of viability, statistically significant stimulation of progesterone by LH or estradiol by FSH was required in each experiment, before overall results were accepted. Cell suspensions were adjusted to allow plating at a density of 200,000 cells per well in Corning 24-well plates. Cells were allowed to attach for at least 24 h. Except where indicated, the medium for experiments where progesterone was measured was Ham's F-10 medium containing 0.5 μM cholesterol. The medium for estradiol measurements was a modification of the Sertoli cell medium described by Padmanabhan *et al.* (11) consisting of a 1:1 mixture of Ham's F-10 and Dulbecco's modified Eagle's medium, 0.1% BSA, 20 μg/mL thyroxine, 10 nM hydrocortisone, 10 ng/mL epidermal growth factor, 0.1 mM methylisobutylxanthine, 10 μM retinol acetate, 5 μg/L transferrin, 20 ng/mL gentamicin, 1 ng/mL Fungizone, and 2.5 μM androstenedione.

Hormone assays. Progesterone was determined by double antibody RIA using kits purchased from ICN (Costa Mesa, CA) or Inkstar (Stillwater, MN) The assay sensitivity was 0.6 nM, and the intra- and interassay coefficients of variation were 8.6 and 14.6%, respectively. Estradiol was determined by RIA, using charcoal to separate bound from free hormone, as described previously (12). The assay sensitivity averaged 92 pmol/L and the intra- and interassay coefficients of variation were 9.7 and 18.4%, respectively. Estradiol was iodinated by the Assay Development Core of the Reproductive Sciences Program of the University of Michigan.

Statistics. For each treatment well, estradiol or progesterone concentrations were determined twice and averaged. The values for each treatment or hormone concentration in an individual experiment represents the mean ± SE of four separate wells of cells. Each well was treated with 1 mL of medium so that results expressed per mL also represent steroid production per 200,000 cells. All data underwent logarithmic transformation before analysis. Analyses were by one-way repeated measures analysis of variance or factorial analysis of variance as appropriate. A P value of < 0.05 was considered significant. Results shown are for individual experiments. Each experiment was repeated at least once to confirm the reproducibility of the findings.

RESULTS

Absence of GH effect on progesterone production. Cells incubated for 24 or 48 h in medium containing 10% FCS, 24 h of serum-free medium, and 24 h with 0.1 to 1000 ng/mL LH exhibited a maximum dose response of 100 ng/mL (data not shown). Subsequent experiments were conducted with 100 ng/mL LH. The time course of incubation required to maximize progesterone release into the medium was determined by preincubating luteinized granulosa cells for 24–120 h in Ham's F-10 medium containing 10% FCS followed by an additional 24 h in serum-free Ham's F-10 medium containing 0.1% BSA and 0.5 μM cholesterol. Cells were then treated for 24 h with vehicle, 100 ng/mL LH, 500 μg/L GH, or a combination of LH and GH. After 24 h, the medium was removed and assayed for progesterone. The results of one representative experiment are shown in Figure 1. After 48 h of preincubation in medium containing serum, the LH-stimulated progesterone accumulation in the medium was significantly greater than that seen in cells treated with vehicle. In the experiment shown in Figure 1, 500 μg/L GH did not stimulate progesterone accumulation above control values either alone or in the presence of LH, at any time point. In a series of experiments, cells were incubated for 24–120 h in medium containing serum followed by a 24-h incubation in serum-free medium containing 0.1% BSA and 0.5 μM cholesterol. The cells were treated with GH in concentrations ranging from 0.1 to 1000 μg/L for an additional 24, 48, or 72 h. In no case did GH increase progesterone synthesis above that seen in cells treated with a control vehicle. A typical experiment demonstrating the lack of a GH dose response on

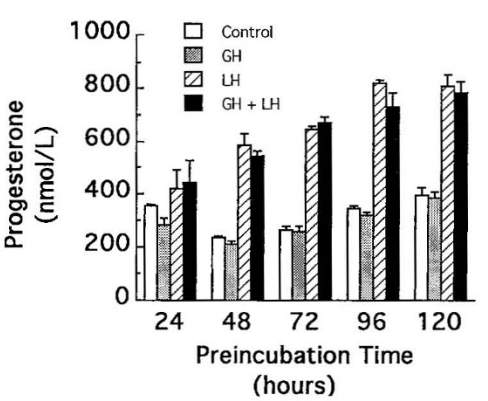

Figure 1. Effect of time in culture on response to GH and LH stimulation of progesterone production. Cells were maintained in culture with Ham's F-10 medium containing 10% FCS for the indicated times. The medium was replaced with serum-free medium for 24 h and then replaced with medium containing a control vehicle, 500 μg/L GH, 100 ng/mL LH, or a combination of 500 μg/L GH and 100 ng/mL LH. After 24 h of hormone exposure, the medium was removed and the progesterone content determined. Each bar is the mean ± SE of four determinations. Each well contained 200,000 cells and 1 mL of medium. Cells treated with LH or LH and GH had significantly more progesterone ($p < 0.01$) than control cells at 48, 72, 96, and 120 h of preincubation with serum. There were no differences in progesterone production between cells treated with control or with GH and between cells treated with LH and LH + GH. This experiment was replicated three times.

progesterone accumulation is shown in Figure 2. In the experiment shown, 200,000 cells/well were preincubated for 48 h with Ham's F-10 medium containing 10% FCS. After a 24-h incubation in serum-free medium, hormones were added for an additional 24 h.

Also shown in Figure 2 are the effects of 100 ng/mL IGF-I on progesterone accumulation with and without GH. IGF-I alone did not increase progesterone accumulation. GH and IGF-I in combination were no more effective than either hormone alone. LH, 100 ng/mL, stimulated progesterone accumulation, and its effect was not augmented by GH. IGF-I has been tested in concentrations ranging from 0.5 to 500 ng/mL without evidence of consistent stimulation of progesterone accumulation (data not shown). The combination of 50 or 500 μg/L GH with submaximal stimulatory concentrations of LH (0.1–10 ng/mL) also failed to further augment LH stimulation of progesterone accumulation (data not shown).

GH effects on estradiol production. The medium of cells treated with Ham's F-10 medium and 0.5 μM cholesterol in cells subjected to conditions described above failed to demonstrate measurable estradiol. Medium 199, Ham's F-10 medium containing 2.5 μM androstenedione, and McCoy's medium also either failed to improve the ability to measure estradiol or lacked evidence of gonadotropin stimulation of estradiol accumulation (data not shown). Therefore, cells were incubated using a modification of the Sertoli cell medium described by Padmanabhan *et al.* (11), where insulin was omitted from the medium formulation to allow detection of IGF-I effects. Cells were maintained in Sertoli cell medium containing 10% FCS for up to 8 d. Before addition of hormones, medium was replaced with serum-free Sertoli cell medium for 24 h. The serum-free medium was replaced with Sertoli cell medium containing vehicle or 10 ng/mL FSH, and after an additional 24 h the medium was removed and assayed for estradiol. Stimulation of estradiol accumulation by FSH was seen for cells maintained for up to 5 d in medium containing serum. After 6 d with serum, or 8 d of total culture, there was no longer FSH stimulation of estradiol accumulation compared with cells treated with vehicle (data not shown). Additionally, the maximum sensitivity of the cells to FSH was determined by incubation with FSH at concentrations of 0.01 to 100 ng/mL The peak estradiol accumulation occurred between 10 and 100 ng/mL FSH (data not shown). Subsequent experiments were performed with 10 ng/mL FSH. Addition of GH at concentrations of 0.1–1000 μg/L did not increase estradiol accumulation in the medium under any condition of incubation (data not shown).

To determine whether GH or FSH might maintain the ability of cells to produce estradiol, cells were first incubated for 24 h in Ham's F-10 medium containing 10% FCS and then placed in serum-free Sertoli cell medium containing a control vehicle, 500 μg/L GH, 100 ng/mL LH, 10 ng/mL FSH, or combinations of GH and LH or FSH. The medium was then removed daily and replaced by fresh Sertoli cell medium containing the same hormones for up to 120 h. Estradiol was measured in the medium collected each day. As shown in Figure 3, estradiol was readily detected in the medium after the first 24 h of incubation in Sertoli cell serum-free medium. However, the stimulation of estradiol accumulation in the medium by 100

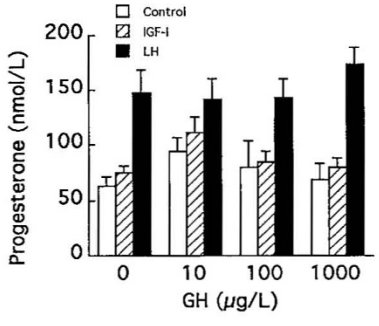

Figure 2. Effect of GH and IGF-I on progesterone production. Cells were incubated for 3 d with Ham's F-10 medium, 24 h with serum-free medium, and 24 h with control vehicle, 100 ng/mL IGF-I, or 100 ng/mL LH and the indicated concentration of GH. After 24 h, the medium was removed and assayed for progesterone concentration. Although LH stimulated progesterone production compared with control cells at each GH concentration tested ($p < 0.05$), the presence of any concentration of GH did not enhance LH-stimulated progesterone production. IGF-I did not increase progesterone production in the absence or presence of GH. Each bar is the mean ± SE of four determinations. Each well contained 200,000 cells and 1 mL of medium. This experiment was replicated once.

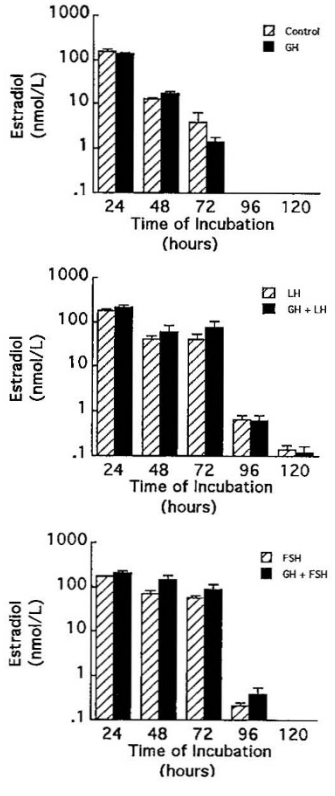

Figure 3. Effect of GH, FSH, and LH on estradiol production. Cells were maintained for 24 h with Ham's F-10 medium containing 10% FCS. The medium was replaced with serum-free Sertoli cell medium containing control vehicle, 500 μg/L GH, 100 ng/mL LH, 10 ng/mL FSH, or a combination of LH and GH or FSH and GH. Medium was collected at the end of each 24 h period for 120 h and replaced with fresh medium containing the same hormones. Estradiol concentration was determined in the medium collected each day. At 48 h, cells treated with GH and FSH produced more estradiol ($p < 0.05$) than did cells treated with FSH alone. Each bar is the mean ± SE of four determinations. Each well contained 200,000 cells and 1 mL of medium. This experiment was replicated three times.

ng/mL LH or 10 ng/mL FSH was not statistically significant presumably because of the lingering effects of FCS constituents present in the preceding 24 h. The medium content of estradiol declined significantly between 24 and 48 h and was virtually unmeasureable at 96 h for cells treated with control vehicle or 500 μg/L GH. Addition of LH to the medium maintained the estradiol content in the medium at greater concentrations than occurred in the absence of LH, but the combination of LH and GH was no more effective than LH alone. FSH also maintained greater concentrations of estradiol in the medium at 48 and at 72 h compared with control values. Cells treated with a combination of GH and FSH averaged 83% more estradiol in the medium than did cells treated with FSH alone. This finding has been replicated in two additional experiments, but when basal estradiol concentrations have been 2–3-fold greater than in the experiment shown in Figure 3, the augmentation of estradiol accumulation in the medium by the combination of GH and FSH compared with FSH alone was first seen at 72 h rather than at 48 h (data not shown). In one experiment, there was no augmentation of the combination of GH and FSH. In no experiment has there been significant stimulation or suppression of estradiol accumulation by GH alone at any concentration (from 0.1 to 1000 μg/L).

The ability of IGF-I to stimulate the accumulation of estradiol in the medium of luteinized granulosa cells was also determined. Cells were incubated after 24 h in Ham's F-10 medium containing 10% FCS. The medium was replaced with Sertoli cell medium containing 500 μg/L GH or 10 ng/mL FSH with or without 100 ng/mL IGF-I. Medium was removed daily and replaced with fresh medium and hormones. After 24 h of incubation there was no difference in estradiol concentration in the cells with any hormone treatment (data not shown). In contrast to the results seen with GH, at 48 h IGF-I produced a 5.6-fold of estradiol accumulation in the medium compared with untreated cells (Fig. 4). The combination of IGF-I and FSH produced a 2.9-fold increase in estradiol accumulation compared with FSH alone. IGF-I and GH in combination were no more effective than IGF-I alone (Fig. 4).

DISCUSSION

The mechanisms by which GH produces its action in target tissues are incompletely understood. The growth-promoting

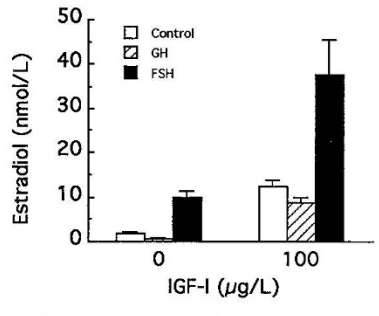

Figure 4. Effect of IGF-I, GH, and FSH on estradiol production. Cells were cultured as described in Figure 3 with control vehicle, the indicated concentration of IGF-I, and 500 μg/L GH or 10 ng/mL FSH. The 48-h time point is shown. IGF-I stimulated estradiol production ($p < 0.05$), and the combination of IGF-I and FSH was more effective than either IGF-I or FSH alone ($p < 0.01$). This experiment was replicated once.

actions of GH appear to be produced via stimulation of production of IGF-I either locally or in peripheral tissues such as liver with subsequent circulation to target sites (13, 14). The metabolic actions of GH are opposite to those of IGF-I and are likely to be produced independently of IGF-I (15). The mechanisms by which GH produces cell differentiation, as in 3T3-F442A cells, are not known, but probably involve the regulation of early transcription factors (16). Thus GH effects in granulosa cells potentially could be produced by an independent effect of GH on cell transcription or may be produced by increasing plasma or local concentrations of IGF-I which then increase steroidogenesis and augment gonadotropin action.

In animal studies, GH appears to produce an increase in steroidogenesis and cell differentiation in granulosa cells (9, 10, 17, 18). However, many of the species studied have local intra-ovarian systems which produce IGF-I as has been demonstrated in rabbit and rat models (7, 19). It has been shown recently that, unlike in animals, human granulosa cells do not express mRNA for IGF-I (20). Therefore, if GH produces direct effects in human granulosa cells, the effects are likely to be governed by direct actions of GH rather than via an IGF-I intermediary.

The results of the present experiments indicate that GH, alone, does not increase estradiol or progesterone synthesis in luteinized granulosa cells. IGF-I, in contrast, produces a brisk increase in estradiol production, as others have found (7). The increase in estradiol production is likely to occur in response to induction of aromatase as described by Christman *et al.* (21).

Our results contrast with those of Barreca *et al.* (22), who have suggested that GH alone can augment granulosa cell estradiol production. The cells used in that report were from women undergoing similar *in vitro* fertilization procedures as in our report, but the luteinized granulosa cells were not subjected to Ficoll separation. Because the GH effects on estradiol production observed in the study of Barreca *et al.* could be abolished by antibodies that prevent IGF-I receptor interaction, it is possible that our Ficoll separation step removed cells that made locally available IGF-I in the ovary. IGF-I production in response to GH administration in these cells could have increased the estradiol production observed in the study of Barreca *et al.* This is particularly likely in view of the observation that human granulosa cells do not express mRNA for IGF-I (20).

In our study and in the study by Carlsson *et al.* (8), the combination of GH and FSH increases estradiol production more than does FSH alone. Even though the results observed in our study were inconsistent, the combination of our data with other reports suggests that GH could play a modest role in enhancing FSH action in granulosa cells. The GH effect has been modest and seen in only carefully controlled conditions compared with the robust estradiol response to IGF-I. Thus, it seems likely that, *in vivo*, GH produces its effects on ovarian granulosa cell steroidogenesis indirectly by increasing circulating IGF-I. Alternatively, GH might increase local IGF-I production from cells in the ovary other than granulosa cells.

There are suggestions that granulosa cells from follicles obtained during surgical oophorectomy or during natural cycle oocyte retrieval may be more responsive to GH than are cells

obtained from women undergoing exogenous gonadotropin administration (8, 23). This seems unlikely in that the cells retrieved in our study exhibit similar magnitude of GH response with FSH as seen in other studies. It is also possible that prepubertal ovarian cells might respond to GH with more differentiation than can be observed in the relatively more differentiated cells obtained during *in vitro* fertilization. Although this possibility cannot be excluded, we have not observed a GH-induced increase in gonadotropin sensitivity, when we have had the opportunity to examine cells from prepubertal girls (our unpublished observations).

Our studies have concentrated on GH and IGF-I effects on ovarian steroidogenesis. GH may have other effects on ovarian cells. Fore example, GH may increase IGFBP-3 concentrations either through local or systemic effects. IGFBP-3 production in the circulation is controlled in large part, by GH (24). IGFBP-3 has anti-gonadotropin-like activities and may thereby have a role in control of ovarian steroidogenesis (25).

Our studies indicate that IGF-I alone can stimulate estradiol production by human luteinized granulosa cells whereas GH alone is ineffective. The combination of IGF-I and FSH is much more effective in stimulation of estradiol production than is the combination of GH and FSH. It therefore seems likely that the predominant mechanism by which GH augments gonadotropin action in the human ovary is by peripheral expression of IGF-I in such tissues as liver followed by circulatory transfer of IGF-I to the ovary where it subsequently augments gonadotropin action. We speculate that the role that GH plays in augmenting pubertal development is mediated by IGF-I action rather than by direct effects of GH at the level of the human ovary.

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