Endocrine, vol. 7, no. 2, 157–163, October 1997 0969-711X/97/7:157-163/$9.75

Effects of GH on IGF-II-Induced Progesterone Accumulation by Cultured Porcine Granulosa Cells

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A total of seven experiments were conducted to investigate the potential facilitative interaction of growth hormone (GH) and insulin-like growth factor II (IGF-II) in stimulating steroidogenesis by cultured porcine granulosa cells and to examine the possible nature of this action. Porcine granulosa cells were cultured in serum-free medium in the presence or absence of GH or prolactin, with or without IGF-II or IGF-I. IGF-II by itself dose (with peak progesterone production of 498 ng/mg DNA/24 h being observed at 100 ng of IGF-II/mL) and time- (with minimum time requirement of 24–48 h) dependently increased progesterone accumulation ($P < 0.01$). Neither GH (dose range 0, 5, 10, 50, 100, and 150 ng/mL) nor prolactin (dose range 0, 0.01, 0.1, 1, 5, 10 μg/mL) alone stimulated progesterone accumulation when compared with the control ($P > 0.05$). However, in the presence of IGF-II, GH proved to be a potent amplifier of IGF-II in progesterone production ($P < 0.01$) with a minimum GH time requirement of 24–48 h. In contrast, prolactin did not influence IGF-II-induced progesterone accumulation ($P > 0.05$). An inhibitory study showed that the presence of cycloheximide (3 μg/mL) or actinomycin D (1 μg/mL) blocked both the stimulatory effect of IGF-II on progesterone accumulation and the amplification of GH on IGF-II induced production ($P > 0.01$), suggesting GH amplification of IGF-II-induced progesterone accumulation is a process involving gene transcription and translation. Northern blot analysis further demonstrated that GH amplification of IGF-II-induced steroidogenesis can be attributed, at least partially, to enhanced IGF-II-induced cytochrome P450 cholesterol side-chain cleavage mRNA by GH.

Key Words: Granulosa cells (porcine); progesterone; GH; IGF-II; cytochrome P450 cholesterol side-chain cleavage mRNA.

Received August 27, 1996; Revised February 18, 1997 and May 5, 1997; Accepted May 14, 1997.

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Introduction

Granulosa cells are well-recognized sites of insulin-like growth factor I (IGF-I) action and receptor expression *(1)*. Evidence derived from in vitro studies indicates that IGF-I augments the steroidogenic response of ovarian granulosa cells to gonadotropic stimulation *(2)*. Although the mechanisms by which insulin-like growth factors (IGFs) influence ovarian function are not known, they are believed to be important regulators of granulosa cell growth and differentiation in the developing ovarian follicle *(3–7)*. Our recent investigations demonstrated that growth hormone (GH) and IGF-I increase progesterone synthesis synergistically in porcine cultured granulosa cells *(8)*. This effect was shown to be time- and dose-dependent and specific to GH.

The effects of insulin-like growth factor-II (IGF-II) in the ovary have not been investigated in equivalent detail. The IGF-II gene is expressed in rat and porcine ovaries *(9,10)*. Human granulosa cells express the IGF-II gene as well as IGF-I and IGF-II receptors *(1)*. Studies have indicated that nanomolar concentrations of IGF-II enhance progesterone *(8,11,12)* and estradiol *(11)* synthesis in cultured porcine granulosa cells. The present study was conducted to investigate the interaction between GH and human recombinant IGF-II on progesterone synthesis in cultured porcine granulosa cells. The mechanisms of action of GH and IGF-II and their effect on the levels of the cytochrome P450 cholesterol side-chain cleavage (P450scc) mRNA were also investigated.

Results

Effect of GH or Prolactin on IGF-II-Induced Progesterone Accumulation (Experiment 1)

In this experiment, IGF-II dose dependence and the effect of GH or prolactin on IGF-II-induced progesterone production were investigated. Porcine granulosa cells were cultured for 96 h in the absence or presence of increasing concentrations of rIGF-II (0, 3, 10, 30, 100, and 150 ng/mL) with or without pGH (0 or 100 ng/mL) or prolactin (0 or 600 ng/mL). IGF-II dose-dependently increased ($P < 0.001$) progesterone production with a minimal dose requirement of 10 ng/mL and a maximal dose of 100 ng/mL (Fig. 1). In

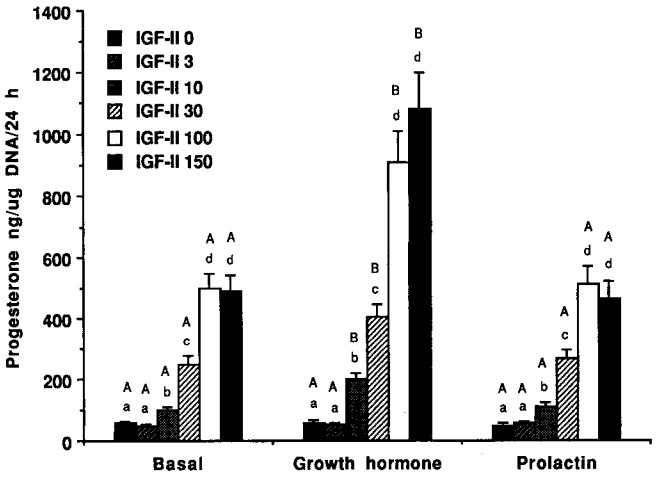

Fig. 1. Effect of GH (0 or 100 ng/mL) or prolactin (0 or 600 ng/mL) on basal or IGF-II-induced (0, 3, 10, 30, 100, and 150 ng/mL) progesterone accumulation (Experiment 1). Small letters refer to comparisons between IGF-II doses (0, 3, 10, 30, 100, and 150 ng/mL) within a treatment. Capital letters refer to comparisons among treatments (basal, GH, and prolactin) within the same dose of IGF-II. Bars with different letters are significantly different ($P < 0.05$).

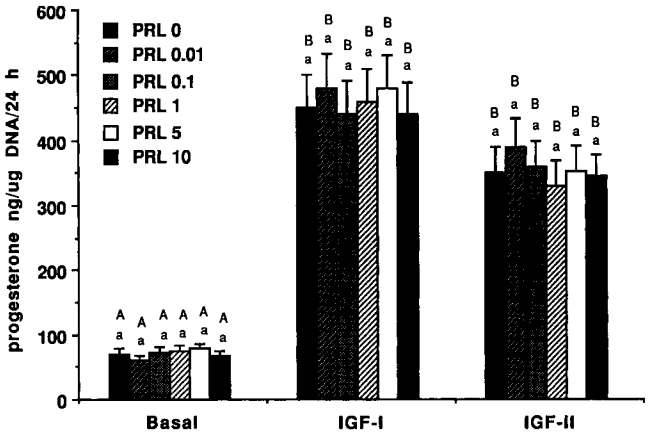

Fig. 2. Effect of prolactin (0, 0.01, 0.1, 1, 5, 10 μg/mL) on IGF-I- (0 or 100 ng/mL) or IGF-II- (0 or 100 ng/mL) induced progesterone accumulation (Experiment 2). Small letters refer to comparisons between prolactin (PRL) doses (0, 0.01, 0.1, 1, 5, and 10 μg/mL) within a treatment. Capital letters refer to comparisons among treatments (basal, IGF-I, and IGF-II) within the same dose of prolactin. Bars with different letters are significantly different ($P < 0.05$).

the absence of IGF-II, the basal accumulation of progesterone remained unchanged in response to treatment with GH or prolactin. However, concurrent treatment with GH resulted in a further ($P < 0.05$) increase in IGF-II-induced progesterone accumulation. In contrast, under the same culture conditions, prolactin lacked the ability to stimulate IGF-II-induced progesterone production ($P > 0.05$).

Effect of Prolactin on IGF-I- or IGF-II-Induced Progesterone Accumulation (Experiment 2)

In this comparison study, the effects of IGF-I or IGF-II and combinations of IGF-I or IGF-II with prolactin on progesterone production were examined. Porcine granulosa cells were cultured for 96 h in the absence or presence of increasing concentrations of prolactin (0, 0.01, 0.1, 1, 5, 10 μg/mL), with or without IGF-I (0 or 100 ng/mL) or IGF-II (0 or 100 ng/mL). Both IGF-I and IGF-II significantly ($P < 0.001$) stimulated progesterone production (Fig. 2). Prolactin, at the doses used in this experiment, had no effect on either basal, IGF-I-induced, or IGF-II-induced progesterone production ($P > 0.05$).

Effect of GH on IGF-I- or IGF-II-Induced Progesterone Accumulation (Experiment 3)

This experiment was designed to investigate the effects of GH on IGF-I- or IGF-II-induced progesterone production. Granulosa cells were cultured for 96 h in the absence or presence of increasing doses of GH (0, 5, 10, 50, 100, and 150 ng/mL), with or without IGF-I (0 or 100 ng/mL) or IGF-II (0 or 100 ng/mL). Progesterone production was dramatically ($P < 0.001$) stimulated by both IGF-I (sixfold of control) and IGF-II (5.6-fold of control). GH by itself induced no stimulation in progesterone accumulation at the

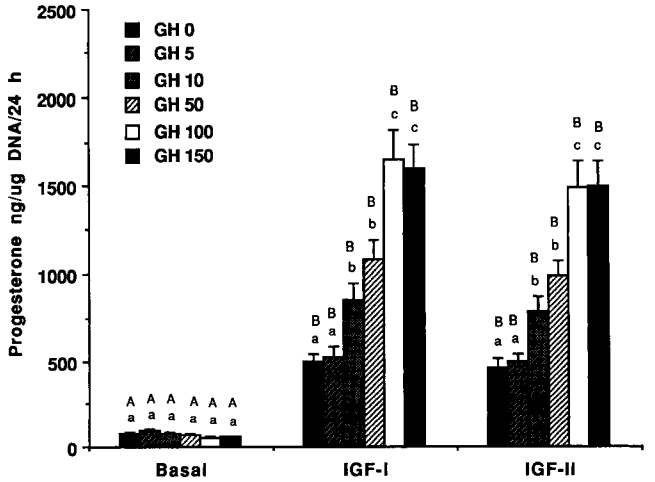

Fig. 3. Effect of GH (0, 5, 10, 50, 100, and 150 ng/mL) on IGF-I- (0 or 100 ng/mL) or IGF-II-induced (0 or 100 ng/mL) progesterone accumulation (Experiment 3). Small letters refer to comparisons between GH doses (0, 5, 10, 50, 100, and 150 ng/mL) within a treatment. Capital letters refer to comparisons among treatments (basal, IGF-I, and IGF-II) within the same dose of GH. Bars with different letters are significantly different ($P < 0.05$).

concentration range tested (0–150 ng/mL). However, in the presence of IGF-I and IGF-II, GH dose-dependently stimulated progesterone production with a GH maximal responsive dose of 100 ng/mL for both IGF-I and IGF-II (Fig. 3).

GH Amplification of IGF-II-Induced Progesterone Accumulation: GH Time Dependence (Experiment 4)

In this GH time-dependence study, the time requirement of GH amplification of IGF-II-induced progesterone accumulation was examined. Granulosa cells were cultured in the absence or presence of GH (0 or 100 ng/mL for 0, 24,

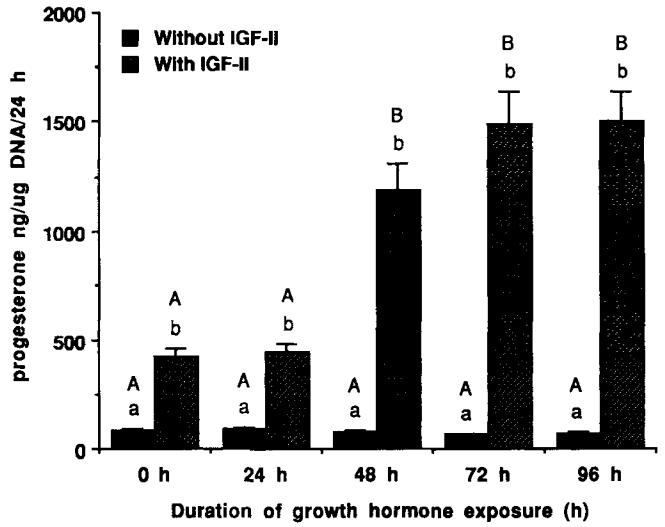

Fig. 4. Time-dependent effect of GH (100 ng/mL for 0, 24, 48, 72, and 96 h) on IGF-II-induced (0 or 100 ng/mL for 96 h) progesterone accumulation (Experiment 4). Small letters refer to comparisons between IGF-II doses (0 or 100 ng/mL) within the same duration of GH exposure. Capital letters refer to comparisons among the different durations of GH exposure (0, 24, 48, 72, and 96 h) within the same dose of IGF-II. Bars with different letters are significantly different ($P < 0.05$).

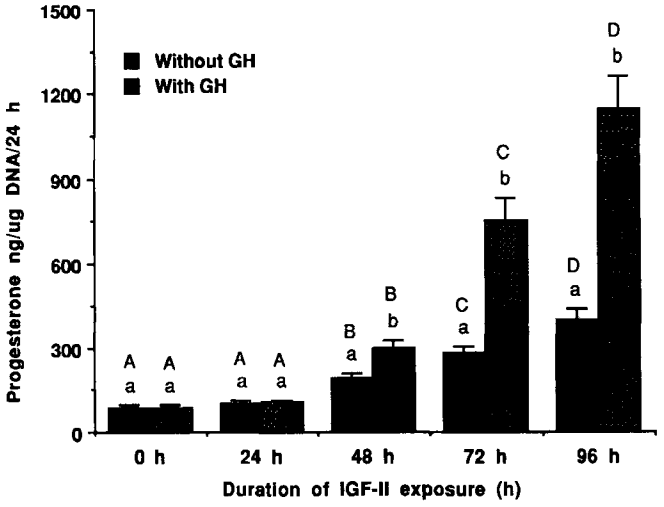

Fig. 5. Time-dependent effect of IGF-II (0 or 100 ng/mL for 0, 24, 48, 72, and 96 h) in the absence or presence of GH (0 or 100 ng/mL for 96 h; Experiment 5). Small letters refer to comparisons between GH doses (0 or 100 ng/mL) within the same duration of IGF-II exposure. Capital letters refer to comparisons among the different durations of IGF-II exposure (0, 24, 48, 72, and 96 h) within the same dose of GH. Bars with different letters are significantly different ($P < 0.05$).

48, 72, or 96 h), with or without IGF-II (0 or 100 ng/mL for 96 h). There was no response difference among the granulosa cells exposed to GH for different periods of time when GH was administrated alone (Fig. 4). Concurrent treatment with IGF-II induced an increase ($P < 0.01$) in progesterone accumulation at 48, 72, and 96 h of exposure to GH.

GH Amplification of IGF-II-Induced Progesterone Accumulation: IGF-II Time Dependence (Experiment 5)

In this experiment, IGF-II time dependence of GH-amplified progesterone accumulation was explored. Granulosa cells were exposed to IGF-II (0 or 100 ng/mL) for 0, 24, 48, 72, and 96 h, with or without GH (0 or 100 ng/mL) for 96 h. IGF-II alone induced an increase ($P < 0.01$) in progesterone accumulation at 48, 72, and 96 h of exposure (Fig. 5). In the presence of GH, the IGF-II-induced progesterone accumulation was 1.6-, 2.7-, and 2.9-fold greater than without GH at 48, 72, and 96 h.

GH Amplification of IGF-II-Induced Progesterone Accumulation: Cycloheximide or Actinomycin D Inhibitory Study (Experiment 6)

To investigate the possible mechanisms subserving GH-amplified IGF-II-induced progesterone production, an inhibitory study was conducted by taking advantage of two inhibitors, a protein synthesis inhibitor, cycloheximide, and an RNA synthesis inhibitor, actinomycin D. Porcine granulosa cells were cultured for 96 h in the absence or presence of GH (0 or 100 ng/mL), IGF-II (0 or 100 ng/mL), with or without cycloheximide (3 μg/mL), or actinomycin D (1 μg/mL) for 24 h. The results revealed that the effects of IGF-II on progesterone production were dependent on protein and/or RNA synthesis (Fig. 6).

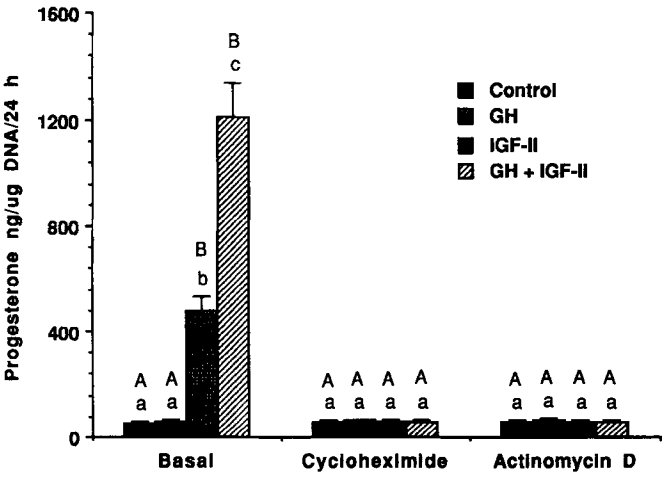

Fig. 6. Inhibitory effects of cycloheximide (3 μg/mL) or actinomycin D (1 μg/mL) on the synergism between GH and IGF-II (Experiment 6). Small letters refer to comparisons among control, GH, IGF-II, and the combination of GH and IGF-II within treatment. Capital letters refer to comparisons among treatments (basal, cycloheximide, and actinomycin D) within control, GH, IGF-II, or the combination of GH and IGF-II. Bars with different letters are significantly different ($P < 0.05$).

Effect of GH on IGF-II-Induced Expression of the Gene Encoding Cytochrome P450 Side-Chain Cleavage (Experiment 7)

In this experiment, the effect of GH on IGF-II-induced expression of the gene encoding P450scc was investigated using the Northern Blot technique. Granulosa cells were cultured at a density of 8×10^6 viable cells/well in a 20 × 100 mm tissue-culture dish in 10 mL of culture media in the

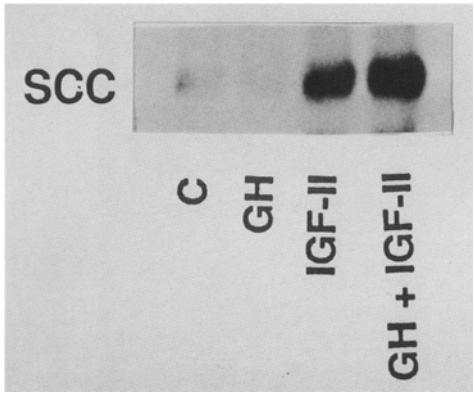

Fig. 7. Effect of pGH (0 or 100 ng/mL) on basal and IGF-II-induced (0 or 100 ng/mL) cytochrome P450scc mRNA levels (Experiment 7).

absence or presence of GH (100 ng/mL), IGF-II (100 ng/mL) or their combination for 96 h. Total RNA (5 μg) was analyzed by Northern Blot for P450scc and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. IGF-II induced a stimulation of 3.2-fold, over the control levels, in the P450scc mRNA levels measured by Northern blot analyses (Fig. 7). Porcine GH (100 ng/mL) alone, induced no stimulation in the P450scc mRNA level. However, when granulosa cells were cultured in the presence of GH, IGF-II induced a 4.8-fold increase, over the control, in the levels of P450scc mRNA.

Discussion

Although a number of physiological studies have implicated GH in altering ovarian function in mammals *(13–17)*, there are relatively few experiments investigating its mechanism of action. It has been suggested that the effects of GH in the ovary involve an elevation in the levels of IGF-I in both the serum of humans *(18)* and in the follicular fluid in pigs *(19)*. These observations lend credence to the idea that the effects of GH are mediated by the stimulation of IGF-I production, which potentiates gonadotrophin action *(20)*. Our previous studies, conducted in porcine cultured granulosa cells, have suggested that GH and IGF-I have divergent pathways of action *(8)*. GH may not necessarily increase granulosa cell secretion of IGF-I, but it does sensitize the granulosa cell for further IGF-I action. Furthermore, this study demonstrates that the interaction between GH and IGF-I is synergistic *(8)*.

The results of the present study demonstrate that cultured porcine granulosa cells are highly responsive to IGF-II. GH did not have any effect on progesterone production, but IGF-II increased progesterone production in a dose- and time-dependent manner. When GH and IGF-II were coadministered, the production of progesterone was amplified. GH amplification of IGF-II-induced progesterone production was observed in the presence of a maximally responsive dose of IGF-II, suggesting that GH and IGF-II have different pathways of action.

Prolactin and GH receptors belong to the family of single membrane-spanning cytokine/hematopoietin receptors, which utilize the JAK2 signaling pathway *(21,22)*. Prolactin, at a dose of 600 ng/mL, had no amplifying effect on IGF-II-induced progesterone production, suggesting that the observed effect is specific to GH.

Although the mechanisms of IGF-I signaling are well characterized *(23,24)*, the IGF-II signal transduction is still under investigation. Many attempts to delineate the IGF-II signaling pathway have employed specific antibodies that block ligand binding to IGF receptors as well as mutant IGF ligands with altered receptor affinities *(25–27)*. The results of some studies suggest that the IGF-II receptor mediates the mitogenic and proliferative effects of IGF-II *(28–30)*, whereas others favor the conclusion that responses to IGF-II may be mediated through either IGF-I or insulin receptors *(26,31,32)*. Although IGF-I and insulin receptors are glycoproteins that share 60% structural homology, IGF-II binds to insulin receptors with low affinity *(23,33)*. Our present study cannot exclude the possibility that responses to IGF-II may be mediated through either IGF-I or insulin receptors. However, we believe that the observed effects on steroidogenesis are more likely mediated through specific IGF-II receptors, since the IGF-II used in this study has been reported to be 150-fold less effective than IGF-I for binding to IGF-I receptors in porcine granulosa cells *(34)*.

IGFs are normally complexed with specific high-affinity binding proteins *(35)*. The intrafollicular presence of IGF binding proteins (IGFBPs), which are regulated by GH *(36)*, makes the possible interactions more complicated. These binding proteins exist in at least six different entities (IGFBPs 1–6) that differ in their amino acid sequence, degree of glycosylation, and tissue distribution. They bind IGFs with an affinity equal to or even greater than that of the IGF receptors *(33)*. The IGFBPs are believed to play a major role in the regulation of IGF action, and have been reported both to enhance and inhibit IGF action *(37)*. Taken together, the available data illustrate the complex nature of IGF-II ligand–receptor interaction and signal transduction, which will require significantly more investigation to determine whether GH mediates its amplified effect on IGF-II action via its effect on IGFBPs or through another mechanism.

Studies by Garmey et al. *(12)* indicated that IGF-II's mechanisms of action included facilitation of sterol delivery via increased LDL binding and metabolism, concomitantly higher steady-state cellular concentrations of LDL receptor mRNA, and augmentation of sterol utilization in progesterone biosynthesis by increasing levels of P450scc mRNA. However, the mechanisms subserving GH amplification of IGF-II-induced progesterone accumulation are not clear. To explore the mechanisms of IGF-II action further, cultured granulosa cells were exposed to two inhibitors, cycloheximide (a protein synthesis inhibitor)

and actinomycin D (a RNA synthesis inhibitor). Our results revealed that the synergism observed between GH and IGF-II in progesterone accumulation is a process involving gene transcription and/or translation, since cycloheximide or actinomycin D completely blocked the synergism between these two agents, but did not reduce basal progesterone accumulation.

Northern blot analysis indicated that GH amplified IGF-II-induced expression of the gene encoding porcine P450scc. This result could be interpreted to suggest that GH-amplified, IGF-II induction of progesterone accumulation is mediated, at least in part, by an increase in P450scc gene expression. However, the present study does not rule out the possibility that GH mediates its amplifying effect on IGF-II action via effects on IGFBPs or receptors for IGF-I and/or IGF-II, because GH has been shown to regulate IGFBP-3 concentration *(36)* and IGFBP-3 influences bioavailability and bioactivity of IGFs *(38)*.

Immunohistochemical and nucleic acid analyses indicate that the GH receptor is present primarily within the large luteal cell of the bovine ovary *(39)*. Follicles and small luteal cells in the ovary express little mRNA for GH receptors and have undetectable levels of GH receptor protein *(39)*. These observations are in agreement with the results of in vivo experiments in which administration of GH to lactating cows resulted in increased weight of the corpus luteum *(40)* and increased progesterone concentration in plasma *(41,42)*. A comparison of the results of this study with in vivo investigations involving granulosa cells from pigs *(43,44)* suggests that the cultured granulosa cells used in our experiments more closely resemble luteal cells than granulosa cells. It is quite possible that differentiation and partial luteinization of the granulosa cells that occur in culture *(45)* may result in the expression of the GH receptor. If this is the case, it may be speculated that the GH-mediated amplification of IGF-II action operates through a GH receptor mechanism in luteinized cultured granulosa cells.

In conclusion, the present studies indicate that GH amplifies IGF-II-induced progesterone accumulation in a dose- and time-dependent manner in cultured porcine granulosa cells. We propose that GH amplifies IGF-II-induced progesterone accumulation by a stimulation of P450scc gene expression.

Materials and Methods

Reagents

Dulbecco's Minimum Essential Medium (DMEM), penicillin, streptomycin, fungizone, fetal calf serum (FCS), and trypan blue stain were obtained from Gibco (Burlington, Ontario). Cycloheximide, actinomycin D, trypsin, papain type III, calf thymus DNA standard, and the reagents used for RNA preparations and Northern blot analyses were purchased from Sigma (St. Louis, MO). Human recombinant IGF-I and IGF-II were purchased from Amersham Canada Ltd. (Oakville, Ontario). Porcine GH (pGH, USDA pGH-B-1) and porcine prolactin (USDA-pPRL-B-1) were obtained as a gift from the National Hormone and Pituitary Program (Rockville, MD).

Granulosa Cell Cultures

Ovaries of prepubertal gilts were obtained from a local abattoir (Intercontinental Packers, Saskatoon, Saskatchewan Canada). The ovaries were collected into a wide-necked bottle filled with sterilized iced saline and transported to the laboratory. Approximately 10 min elapsed from slaughter to ovary collection. Granulosa cells were obtained by fine-needle aspiration of medium-sized (4–6 mm) nonatretic follicles within 1–1.5 h of ovary collection. Follicles were determined to be nonatretic if they were uniformly translucent and vascularized.

After collection, granulosa cells were washed three times in DMEM containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL fungizone, and recovered by centrifugation (10 min at 200*g*). Viable granulosa cells, determined by trypan blue exclusion *(8)*, were plated in plastic cell-culture plates (Falcon, Lincoln Park, NJ).

For the experiments involving progesterone production, cells were plated at 2×10^6 viable cells/well in 24-well plates in 1 mL of culture media. For Northern blot analyses, cells were plated at 8×10^6 viable cells/well in 10-mm plates in 10 mL of culture media.

Cell cultures were maintained in a CO_2 incubator (Forma Scientific Inc. Marietta, OH) at 37°C under a water-saturated atmosphere of 95% air and 5% CO_2. Cells were initially cultured in serum-containing (10% FCS) DMEM for 24 h to allow cell attachment to the plates. At 24 h, serum-containing DMEM was discarded and then washed twice using serum-free DMEM. After washing, cells were cultured for an additional 96-h period in serum-free DMEM, in the absence or presence of treatment agents (pGH, IGF-I, IGF-II, prolactin, and their combinations) as required by each experimental design (for details, *see* Results section). At the conclusion of this period, culture medium was discarded, and the cells were washed twice with serum-free DMEM and reincubated for an additional 24 h in the medium (for progesterone assay) or reincubated for the same length of time in the serum-free DMEM. At the end of the experiment (i.e., 144 h after plating), the culture media were collected and stored at –20°C until assayed for progesterone. Progesterone was assayed by radioimmunoassay (RIA) according to the methods described by Rajkumar et al. *(46)*.

Our culture system involved granulosa cell culture for 6 d, with FBS included for the first 24 h to facilitate adherence of the cells to the wall. In a preliminary experiment, freshly harvested granulosa cells and granulosa cells after 6 d of culture both responded to FSH treatments. FSH-induced production of estradiol by the two cell types was comparable. In addition, the estradiol/progesterone ratio was constant over the 6-d period.

A total of seven experiments were conducted following the general culture procedures described in the previous sections. Each experiment was replicated three times with each replicate involving granulosa cells obtained from approx 300 ovaries. Between 2 and 4 medium-sized follicles (4–6 mm) were aspirated from each ovary, and over 900 follicles were used to produce the granulosa cell pool used in any given replicate to minimize the biochemical variation possibly existing among follicles from within and among animals.

Determination of Cellular DNA Content

For cellular DNA assays, granulosa cells were washed twice with calcium- and magnesium-free phosphate-buffered saline solution (PBS) and dispersed by incubation with a solution of 0.05% trypsin–0.05% EDTA for 5 min at 37°C. Trypsin-dispersed cells from six wells were washed three times in PBS, and digested with a solution containing papain type III (125 µg/mL in sterile PBS, pH 6.0), with 5 m*M* cysteine-HCl and 5 m*M* Na_2EDTA *(47)*. DNA was estimated by the fluorometric assay using the bisbenzimidazole fluorescent dye, Hoechst 33258 *(48)*. Fluorescence emission was determined in a Hoeffer TKO-100 DNA fluorometer (Hoeffer, San Francisco CA) using calf thymus DNA standard. This assay has a sensitivity of 6 ng/mL of dye solution.

RNA Preparations and Northern Blot Analyses

RNA was extracted from plates of cells using 2 mL of 1% sodium dodecyl sulfate 10 m*M* EDTA, pH 7.0, solution, and isolated by acid phenol/chloroform extraction *(49)*. RNA samples (5 µg) were denatured, size-fractionated by electrophoresis on 1% agarose-formaldehyde gels, and transferred to nylon blotting membranes (Hybond-N, Amersham Canada Ltd. Oakville, Ontario) by diffusion blotting. RNA was crosslinked to membranes using an ultraviolet (UV) Stratalinker 1800 (Stratagene, La Jolla, CA).

Hybridizations were performed utilizing cDNAs complementary to mRNAs encoding porcine cytochrome P450 side-chain cleavage (P450scc) *(50)* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *(51)*. Probes were labeled by random primer synthesis *(52)*. Membranes were incubated for 16 h at 65°C in a solution containing 1 *M* NaCl, 10% dextran sulfate, and 1% SDS. After hybridization, membranes were washed twice for 15 min at room temperature in 2X SSC–0.5% SDS and twice in 1X SSC–0.5% SSC at 65°C (20X SSC contained 3 *M* NaCl and 0.3 *M* Na_3 citrate). Membranes were first hybridized with labeled side-chain cleavage cDNA. For subsequent hybridization with labeled GAPDH cDNA, filters were stripped by incubation in 10 m*M* Tris–10 m*M* EDTA for 30 min at 90°C.

Northern blot autoradiograms were quantified by computer-aided scanning densitometry using a ScanJet IIp Hewlett Packard scanner and analyzed with a digital image processing program (NIH Image 1.41). Data were corrected for variability in loading by calculation concerning the ratio to GAPDH, which was unaffected by treatment with GH or IGF-I (Xu et al., unpublished data).

Statistical Analyses

All experimental data are presented as means ± SEM, which were obtained in three separate replicates involving six cultures per treatment. Data obtained were subjected to analysis of variance (ANOVA). When a significant *F*-value was present, Fisher's least-significant difference test was used for individual comparison of means *(53)*. Comparisons with $P < 0.05$ were considered significant.

Acknowledgments

This research was supported by the Province of Saskatchewan's Agriculture Development Fund grant to P. A. T. and Medical Research Council of Canada grant to J. C. The authors wish to thank the National Hormone and Pituitary Program, USDA, for the generous gift of porcine GH. We also thank Intercontinental Packers Ltd. for providing porcine ovaries. We also thank Cristine Meaden for the editorial assistance and, Claire Card and Marie Rodway for their helpful comments on the manuscript.

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