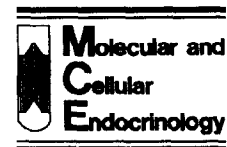




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Growth hormone amplifies insulin-like growth factor I induced progesterone accumulation and P450scc mRNA expression

Y.P. Xu^a, P.J. Chedrese^b, P.A. Thacker^{a,*}

^aDepartment of Animal Science, University of Saskatchewan, Saskatoon, S7N 0W0, Canada

^bDepartment of Obstetrics and Gynecology, University of Saskatchewan, Saskatoon, S7N 0W0, Canada

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Abstract

The interaction of growth hormone (GH) and insulin-like growth factor I (IGF-I) in the acquisition of progesterone biosynthetic capacity were examined in cultured porcine granulosa cells. Basal progesterone production was not affected ($P > 0.05$) by GH treatment. However, concurrent treatment with GH produced a 4.1-fold increase (539 versus 2214 ng/culture) in the IGF-I-stimulated accumulation of progesterone. GH potentiated IGF-I induced progesterone production in a dose and time dependent manner, with a time requirement of 48 h or less. The amplified effect of GH was not attributable to changes in cellular protein, DNA content, cell number, plating efficiency or cell viability. Moreover, Northern blot analyses revealed that GH enhanced IGF-I induced expression of the gene encoding cytochrome P450 side chain cleavage. These observations provide further evidence to support the role of GH in the regulation of ovarian steroidogenesis.

Keywords: Granulosa cell (Swine); Growth hormone; IGF-I; Progesterone; P450 side chain cleavage

1. Introduction

The development of the capacity to biosynthesize progesterone by granulosa cells is of obvious importance to their subsequent function as a constituent of the corpus luteum. The ability of gonadotropins to induce progesterone biosynthesis in granulosa cells is well established (Richards, 1979). However, the modulation of this process by other factors, including insulin-like growth factor I (IGF-I) and growth hormone (GH) remains under investigation (Adashi, 1992; Guidice, 1992).

Traditionally, IGF-I has been viewed as a hepatic product (Lund et al., 1986). Now, it is accepted that IGF-I may also be synthesized in the ovary (Murphy et al., 1987) where it plays autocrine and paracrine regulatory roles (Hammond et al., 1993). Specifically, IGF-I augmented basal (Veldhuis and Furlanetto, 1985), oestradiol- (Veldhuis et al., 1986a) and FSH-mediated (Adashi et al., 1985a) acquisition of progesterone biosynthetic capacity

in granulosa cells. IGF-I also increases the FSH-stimulated induction of LH receptors (Adashi et al., 1985b), basal (Erickson et al., 1989) and FSH-induced aromatase activity (Adashi et al., 1985c) and the FSH-stimulated cAMP accumulation (Adashi et al., 1986a) in granulosa cells.

Several lines of evidence have shown that GH also has an important role in the regulation of several ovarian functions, although its exact role is still controversial. GH has been reported to enhance the FSH-induced differentiation of cultured rat granulosa cells (Jia et al., 1986) and is required for sensitization of the ovary to gonadotropins (Sheikholislam and Stemfel, 1972; Ramaley and Phares, 1980). In humans, GH has been used to augment induction of ovulation in patients suffering from hypogonadotropic hypogonadism (Homburg et al., 1988). Our studies have shown that GH induces anoestrus in about 50% of previously cycling gilts (Kirkwood et al., 1987). In addition, daily injection of GH delays puberty in pigs (Bryan et al., 1989). Alternatively, in vitro studies have shown that GH is capable of amplifying IGF-I induced progesterone biosynthesis by cultured porcine granulosa cell (Xu et al., 1993a).

* Corresponding author, Department of Animal Science, University of Saskatchewan, Saskatoon, Canada S7N 0W0. Tel.: +1 306 966 4159; Fax: +1 306 966 4151.

The mechanisms by which GH exerts its actions in the ovary remain unknown. GH enhances FSH-induced LH/hCG receptor activity and steroidogenesis (Jia et al., 1986) and modulates the effects of pituitary gonadotropins on ovarian follicular growth and maturation (Fauser et al., 1988). It has been suggested that the effect of GH in the ovary is mediated by a mechanism involving an elevation in the levels of IGF-I (Homburg et al., 1988). Treatment with GH is associated with a rise in circulating concentrations of IGF-I in humans (Homburg et al., 1990) and in follicular fluid in pigs (Spicer et al., 1992). These observations lend credence to the idea that the effects of GH are mediated by stimulation of IGF-I production in the ovary, and potentiates gonadotropin action (Adashi, 1992). The objective of the present study was to investigate the interaction of GH and IGF-I in the acquisition of progesterone biosynthetic capacity, and their possible mechanisms of action, in a primary culture of porcine granulosa cells.

2. Materials and methods

2.1. Reagents

Dulbecco's minimum essential medium (DMEM), antibiotic-antimycotic mixture, foetal calf serum and trypan blue stain were obtained from Gibco (Burlington, Ontario). Papain, calf thymus DNA, trypsin and the reagents used for RNA preparations and Northern blot analyses were purchased from Sigma (St. Louis, MO). Hoechst 33258 was purchased from Hoeffer (San Francisco, CA). Human recombinant IGF-I was purchased from Amersham Canada Ltd. (Oakville, Ontario). Porcine growth hormone (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).

2.2. Granulosa cell culture

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) non-atretic follicles within 1–1.5 h of ovary collection. Follicles were determined to be non-atretic 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 (200 × g). Viable granulosa cells, determined by trypan blue exclusion (see below), were plated in plastic cell culture plates (Falcon, Lincoln Park, NJ).

For the experiments involving progesterone assays, viable cell counts, DNA or protein determinations, 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 100 × 20 mm tissue culture dish in 10 ml culture media.

Cell cultures were maintained in a CO₂ incubator (Forma Scientific Inc. Marietta, OH) at 37°C under a water saturated atmosphere of 95% air and 5% CO₂. 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 (GH, IGF-I, prolactin and their combinations) as required by each experiment (for details see Section 3 and figure legends). At the conclusion of this period, culture media were discarded and the cells were washed twice with serum-free DMEM and re-incubated for an additional 24 h in the medium. At the end of the experiment (i.e. 144 h after plating), the culture medium was collected and stored at –20°C until assayed for progesterone content. Progesterone was assayed by radioimmunoassay (RIA) according to the method described by Rajkumar et al. (1985).

2.3. Determination of cellular plating efficiency, viability, DNA and protein

Granulosa cells were washed twice with calcium- and magnesium-free phosphate-buffer salt solution and dispersed by incubation with a solution of 0.05% trypsin-0.05% EDTA for 5 min at 37°C. Dispersed cells of six wells were spun down and the cellular pellets were counted in a haemocytometer. Viability was assessed by the trypan blue exclusion test (Campbell, 1979). Plating efficiency and cellular viability were expressed in terms of the percentage of cells attached per well and the percentage of viable cells per well, respectively (Adashi et al., 1985d).

For cellular DNA assays, trypsin-dispersed cells, from six wells, were washed three times in phosphate-buffer salt solution (PBS), and digested with a solution containing papain type III (125 µg/ml in sterile PBS, pH 6), with 5 mM cysteine-HCl and 5 mM Na₂EDTA (Kim et al., 1988). DNA was estimated by the fluorometric assay using the bisbenzimidazole fluorescent dye, Hoechst 33258 (Labarca and Paigen, 1980). 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. Granulosa cells were scraped from the culture well using a rubber policeman. Cellular protein content was determined in 0.1 M NaOH dissolved granulosa cells by the procedure of Lowry et al. (1951).

2.4. RNA preparations and Northern blot analyses

RNA was extracted from plates of cells using 2 ml of 1% sodium dodecyl sulphate 10 mM EDTA (pH 7.0) solution, and isolated by acid phenol/chloroform extraction (Liu et al., 1994). 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) Stratilinker 1800 (Stratagene, La Jolla, CA).

Hybridizations were performed utilizing cDNAs complementary to mRNAs encoding porcine cytochrome P450 side chain cleavage (cytochrome P450_{sc}) (Mulheron et al., 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985). Probes were labelled by random primer synthesis (Feinberg and Vogelstein, 1983) with [α^{32} P]dCTP (>3000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of $(1.5\text{--}3.0) \times 10^9$ dpm/µg DNA. Membranes were incubated for 16 h at 65°C in a solution containing 1 M NaCl, 10% dextran sulphate and 1% SDS. After hybridization, membranes were washed twice for 15 min at room temperature in $2 \times$ SSC–0.5% SDS and twice in $1 \times$ SSC–0.5% SSC at 65°C ($20 \times$ SSC contained 3 M NaCl and 0.3 M Na₃ citrate). Membranes were first hybridized with labelled cytochrome P450_{sc} cDNA. For subsequent hybridization with labelled GAPDH cDNA, filters were stripped by incubation in 10 mM Tris–10 mM EDTA for 30 min at 90°C.

Northern blot autoradiograms were quantitated 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 as the ratio to GAPDH, which was unaffected by treatment with GH or IGF-I.

2.5. Experimental protocol

A total of eight experiments were conducted following the general culture procedures described in the previous sections. Each of the eight experiments was replicated three times with each replicate involving granulosa cells obtained from approximately 300 ovaries. Between 2 and 4 medium sized follicles were aspirated from each ovary and over 900 follicles were used to produce the granulosa cell pool used in any given replicate. For time course studies (experiments 3 and 4), all the groups terminated treatments at the same end-point of 120 h of culture, but started treatment at different time points (i.e. GH or IGF-I added 120, 96, 72, 48, and 24 h after the start of culture).

2.6. Statistical analyses

All experimental data are presented as means \pm 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 (SAS, 1985). Comparisons with *P* < 0.05 were considered significant.

3. Results

In experiment 1 (IGF-I dose-dependence experiment), growth hormone amplification of IGF-I induced progesterone accumulation was investigated. Granulosa cells were cultured for 96 h in the absence or presence of increasing concentrations of IGF-I (5, 10, 25, 50, 100 and 150 ng/ml), with or without pGH (100 ng/ml). Treatment of cultured granulosa cells with graded doses of IGF-I induced dose dependent increases in the accumulation of progesterone to a peak of 539 ng/culture at 100 ng/ml (Fig. 1). Addition of pGH (100 ng/ml) resulted in a significant (*P* < 0.01) potentiation of the IGF-I effect for all the IGF-I doses higher than 10 ng/ml (4, 3.9, 3.9, 4.1 and 4.2 fold increments for 10, 25, 50, 100 and 150 ng/ml of IGF-I, respectively, Fig. 1).

In experiment 2 (GH dose-dependence experiment), the capacity of pGH to potentiate IGF-I-induced progesterone accumulation was further characterized. Granulosa cells were cultured in the presence or absence of IGF-I (50 ng/ml) with or without increasing concentrations (0.1, 0.3, 1.0, 3.0, 10, 30, and 100 ng/ml) of pGH. Porcine GH, at a dose of 100 ng/ml, did not alter progesterone accumulation when it was added alone. Concurrent treatment with IGF-I resulted in a significant increase in progesterone accumulation at pGH doses of 3.0 ng/ml (*P* < 0.01) and higher (Fig. 2).

In experiment 3 (GH time-dependence experiment),

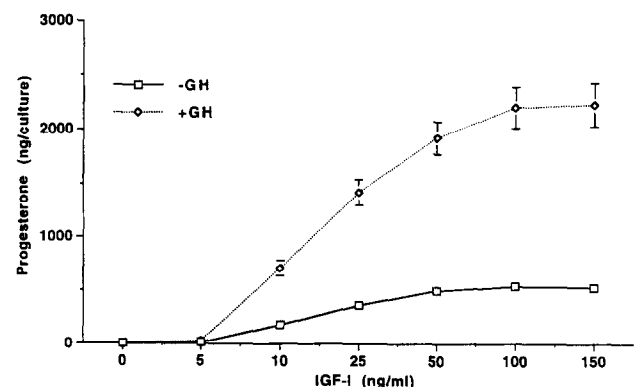


Fig. 1. Effect of pGH on basal and IGF-I-induced progesterone accumulation (experiment 1). In experiment 1 (IGF-I dose-dependence experiment), growth hormone amplification of IGF-I induced progesterone accumulation was investigated. Granulosa cells were cultured for 96 h in the absence or presence of increasing concentrations of IGF-I (5, 10, 25, 50, 100, and 150 ng/ml), with or without pGH (100 ng/ml). The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment.

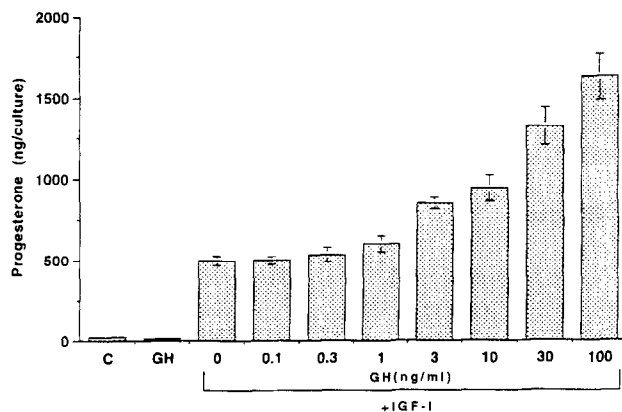


Fig. 2. Dose-dependence effect of pGH on IGF-I-induced progesterone accumulation (experiment 2). In experiment 2, the capacity of pGH to potentiate IGF-I-induced progesterone accumulation was further characterized. Granulosa cells were cultured in the absence (C) or presence of pGH (100 ng/ml). Granulosa cells were also cultured with or without IGF-I (50 ng/ml), and in the presence of increasing concentrations (0.1, 0.3, 1, 3, 10, and 100 ng/ml) of pGH. The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment.

granulosa cells were exposed to pGH (100 ng/ml) for 0, 24, 48, 72, and 96 h, with or without IGF-I (50 ng/ml) for 96 h. Porcine GH, administered alone, did not change progesterone accumulation ($P > 0.05$) at any of the time points studied (Fig. 3). Concurrent treatment with IGF-I induced an increase ($P < 0.01$) in progesterone accumulation at 48, 72, and 96 h of exposure to pGH (Fig. 3).

In experiment 4, IGF-I time dependence of pGH amplified progesterone accumulation was explored. Granulosa cells were exposed to IGF-I (50 ng/ml) for 0, 24, 48, 72, and 96 h, with or without GH (100 ng/ml) for 96 h. IGF-I alone induced an increase ($P < 0.01$) in progesterone accumulation at 48, 72, and 96 h of exposure, respec-

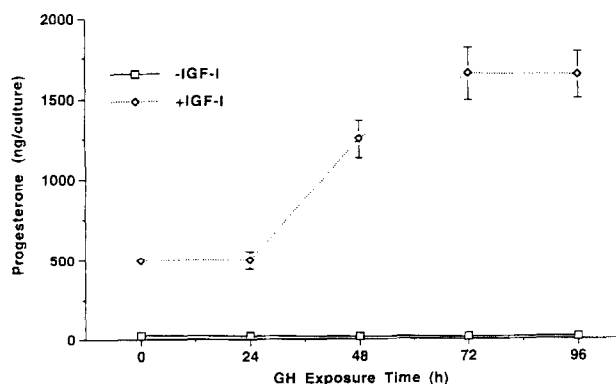


Fig. 3. Time-dependence effect of pGH on IGF-I-induced progesterone accumulation (experiment 3). In experiment 3, time-dependence effect of pGH on IGF-I induced progesterone accumulation was examined. Granulosa cells were cultured in the absence or presence of IGF-I (50 ng/ml), with pGH (100 ng/ml) for 0, 24, 48, 72, and 96 h. The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment.

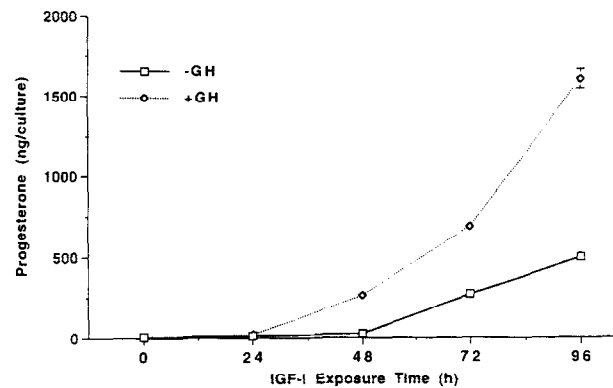


Fig. 4. Time dependent effect of IGF-I on progesterone accumulation in the presence or absence of pGH (experiment 4). In experiment 4, IGF-I time dependence of pGH amplified progesterone accumulation was explored. Granulosa cells were cultured in the absence or presence of pGH (100 ng/ml), with IGF-I (50 ng/ml) for 0, 24, 48, 72, and 96 h. The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment.

tively (Fig. 4). In the presence of pGH, the IGF-I-induced progesterone accumulation was 1.5, 10.6, 2.5, and 3.2 fold increments (+GH over -GH) at 48, 72, and 96 h respectively (Fig. 4).

Since sequence similarity between prolactin and GH receptors has been well documented (Kelly et al., 1993), a cross-effect could be expected between these two hormones. To address GH specificity on the amplification of IGF-I-induced progesterone accumulation in cultured granulosa cells, a specificity study (experiment 5) was conducted using prolactin, instead of pGH. Granulosa cells were cultured in the absence or presence of IGF-I (50 ng/ml), with or without pGH (100 ng/ml) or prolactin (600 ng/ml). Basal progesterone accumulation was not affected by prolactin at the dose of 600 ng/ml (Fig. 5). Concurrent treatment with prolactin did not induce an

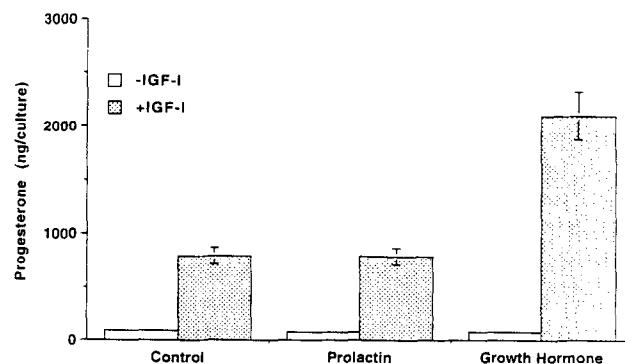


Fig. 5. Effect of pGH, PRL, IGF-I and their combination on progesterone accumulation (experiment 5). Specificity of GH amplification of IGF-I-induced progesterone accumulation in cultured granulosa cells was examined. Granulosa cells were cultured in the absence or presence of IGF-I (50 ng/ml), with or without pGH (100 ng/ml) or PRL (600 ng/ml). The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment.

Table 1

Effect of pGH, IGF-I, and their combination, on progesterone production (experiment 6)

Treatments	Progesterone (ng/ μ g protein)	Progesterone (ng/ μ g DNA)	Progesterone (ng/ 10^6 cells)
Control	0.8 ± 0.03^b	8.9 ± 0.7^a	1.4 ± 0.1^a
GH	0.4 ± 0.02^a	9.4 ± 0.8^a	1.2 ± 0.1^a
IGF-I	27.6 ± 3.1^c	437.5 ± 38.2^b	81.0 ± 11.2^b
GH + IGF-I	94.5 ± 8.6^d	1589.6 ± 95.6^c	269.6 ± 24.7^c

To evaluate whether potentiation of IGF-I-induced progesterone accumulation by pGH could account for changes in cellular protein, DNA content, cell number, granulosa cells (2×10^6 viable cells/ml) were initially cultured for 96 h under serum-free conditions, in the absence or presence of IGF-I (50 ng/ml), with or without GH (100 ng/ml). At the termination of this period, the media were discarded, and the cells were washed and re-incubated for an additional 24 h. Collected media were assayed for their total progesterone content by RIA. Granulosa cells were assayed for protein, DNA and cell numbers as treatment required. The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment. Means followed by different letters are significantly different according to Fisher's test ($P < 0.05$).

increase in IGF-I stimulated accumulation of progesterone ($P > 0.05$).

To evaluate whether potentiation of IGF-I-induced progesterone accumulation by pGH could account for changes in cellular protein, DNA content, cell number, cell viability and plating efficiency, granulosa cells were cultured for 96 h in the presence or absence of IGF-I (100 ng/ml), with or without pGH (100 ng/ml) (experiments 6 and 7). Under these conditions, IGF-I stimulated progesterone production when data were expressed on the basis of unit of protein, DNA and 10^6 cells. Porcine GH by itself had little effect on basal progesterone production

Table 2

Effects of GH, IGF-I and their combination on cell plating efficiency and viability (experiment 7)

Treatments	Plating efficiency (%)	Viability (%)
Control	55.7 ± 5.4	85.4 ± 7.9
GH	51.9 ± 8.1	84.3 ± 8.6
IGF-I	56.4 ± 9.1	81.6 ± 9.2
GH + IGF-I	54.3 ± 7.9	85.1 ± 4.6

To evaluate if potentiation of IGF-I-induced progesterone accumulation by pGH could account for changes in cell viability and plating efficiency, granulosa cells (2×10^6 viable cells/ml) were initially cultured for 96 h under serum-free conditions, in the absence or presence of IGF-I (50 ng/ml), with or without GH (100 ng/ml). At the termination of this period, the media were discarded, and granulosa cells were washed and re-incubated for an additional 24 h. The total number of viable cells in the medium and in the trypsinization solution were counted. Plating efficiency and viability were calculated as described in Section 2. The data are presented as culture progesterone means \pm SEM, which were obtained in three separate replicates involving six cultures per treatment. No means were significantly different according to analysis of variance ($P > 0.05$).

compared with the control. However, in the presence of IGF-I, pGH further stimulated progesterone production by 3.5-, 3.6- and 3.3-fold when data were corrected per μ g of protein, per μ g of DNA or per 10^6 cells (experiment 6; Table 1), respectively. Cell viability and plating efficiency were not affected ($P > 0.05$) by pGH, IGF-I or their combination (experiment 7; Table 2).

In experiment 8, the effect of pGH on basal and IGF-I-induced cytochrome P450_{scc} mRNA abundance was studied. Granulosa cells were cultured for 96 h in the absence or presence of IGF-I (50 ng/ml), with or without pGH (100 ng/ml). Total RNA (5 μ g/ml) was analyzed by

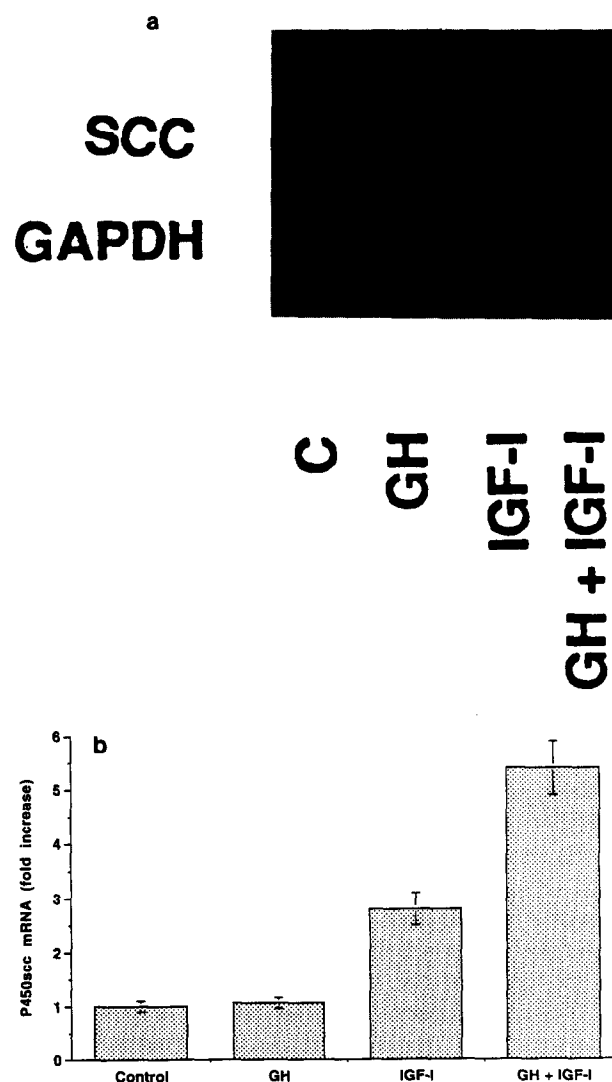


Fig. 6. Effect of pGH on basal and IGF-I-induced cytochrome P450_{scc} mRNA levels (experiment 8). The effect of pGH on basal and IGF-I-induced cytochrome P450_{scc} mRNA abundance was investigated. Granulosa cells were cultured in the absence or presence of IGF-I (50 ng/ml), with or without pGH (100 ng/ml). Total RNA (5 mg) was analyzed by Northern Blot for cytochrome P450_{scc} and GAPDH mRNAs. (a) Representative Northern blot; (b) mRNA levels quantitated by scanning densitometry. Cytochrome P450_{scc} mRNA levels were corrected for hybridization to GAPDH mRNA.

Northern blot for cytochrome P450_{scc} and GAPDH mRNAs. IGF-I (50 ng/ml) induced a stimulation of 2.8-fold, over the control levels, in the cytochrome P450_{scc} mRNA levels measured by Northern blot analyses (Fig. 6). pGH (100 ng/ml) alone induced no stimulation in the cytochrome P450_{scc} mRNA levels. However, when granulosa cells were cultured in the presence of pGH, IGF-I induced a 5.4-fold increase, over the control, in the levels of cytochrome P450_{scc} mRNA.

4. Discussion

The effect of pGH on the IGF-I-induced accumulation of progesterone was studied *in vitro* using primary cultures of porcine granulosa cells. Our observations indicate that GH synergizes with IGF-I in the induction of progesterone biosynthetic capacity in a time- and dose-dependent manner. This study represents what we believe is the first attempt to elucidate the mechanism by which GH influences progesterone accumulation in porcine granulosa cells.

GH has long been recognized as playing an important role in reproductive physiology. In the normal transition to puberty, GH is required for sensitization of the ovary to gonadotropins (Sheikholislam and Stemfel, 1972; Ramaley and Phares, 1980). Delayed puberty, associated with isolated GH deficiencies, is advanced with GH therapy (Sheikholislam and Stemfel, 1972). The amplified effect of GH on IGF-I induced progesterone accumulation reported in the present paper may explain the involvement of GH in pubertal development and the physiological significance of peripubertal elevations of both GH and IGF-I in young women (Apter and Butzow, 1993). These observations may be interpreted to suggest that GH plays a role in promoting juvenile and early follicular development by serving as an amplifier of steroidogenesis or by sensitizing granulosa cells to IGF. In this regard, studies using adipocyte models have demonstrated that GH sensitizes cells for further IGF-I action (Zezulak and Green, 1986).

The involvement of GH in ovarian function has long been recognized (Bartke, 1964; Advis, 1981). However, previous investigators apparently have not been able to distinguish between the potential direct actions of GH on the ovary or the effects of GH-dependent secretion of IGF-I at extraovarian sites. IGF-I is produced by granulosa cells (Hammond et al., 1985) and is under the control of GH (Davoren and Hsueh, 1986). It has been speculated that the ability of GH to promote differentiation of granulosa cells may be due, at least in part, to the stimulation of ovarian IGF-I production with the consequent autocrine amplification of gonadotropins action (Hsu and Hammond, 1987). If GH transmitted its effect on granulosa cell steroidogenesis solely via IGF-I, then it would follow that a culture of granulosa cells which was responding maximally to a saturating level of IGF-I

(100 ng/ml) would show no further response when GH was added.

The additivity experiment done in the present study (Fig. 1) showed that GH had no effect on progesterone accumulation. However, granulosa cells which were responding maximally to a saturating level of IGF-I (100 ng/ml) showed further responses in progesterone accumulation when GH was added (Fig. 1), suggesting that GH and IGF-I have divergent pathways of action. The stimulatory effect of GH on IGF-I induced progesterone accumulation may not be due to its effect on IGF-I gene expression and synthesis in the cultured porcine granulosa cells because, if this was true, then GH by itself should also stimulate progesterone accumulation. Our present study failed to support this hypothesis.

The cellular pathways by which binding of growth hormone to its receptor elicits its diverse effects have eluded investigators for many years. Data from our laboratory have been interpreted to suggest that cultured porcine granulosa cells fail to respond to pGH with an increase in the levels of intracellular cAMP (Xu et al., 1993b). Recently, Janus kinase 2 (JAK2) tyrosine kinase has been identified as a signaling molecule for growth hormone (for review see Carter-Su et al., 1994). Indeed, JAK2 is the first signaling molecule identified that interacts with the growth hormone receptor (Carter-Su et al., 1989). This identification has already allowed researchers to determine that GH promotes the association of kinase with GH receptors, activates the kinase, and promotes phosphorylation of tyrosyl residues on both receptor and kinase (Bazan, 1990; Velazquez et al., 1992). However, how GH sensitizes the cell for further IGF-I action is not clear.

The receptors for prolactin and GH belong to a similar family of single membrane-spanning cytokine/haematopoietin receptors and both GH and prolactin utilize JAK2 as the signaling molecules (Larson et al., 1976; Boutin et al., 1988). The GH receptor shares about 30% sequence identity with the prolactin receptor with about 70% identity in certain domains in the extracellular and intracellular regions (Boutin et al., 1989). Given the similarity of receptors for GH and prolactin and the fact that both of the receptors share a common signaling pathway, one might expect that prolactin may have the same amplifying effect as GH. However, our specificity studies revealed that prolactin, at a dose of 600 ng/ml (Experiment 5), had no amplifying effect on IGF-I induced progesterone accumulation and this was true over a wide range (0.01–100 µg/ml) of prolactin levels tested (Xu, unpublished data), suggesting the synergism between GH and IGF-I in steroidogenesis is a specific process.

The reason why prolactin did not have an effect is unlikely due to the absence of prolactin receptors. Although we have not run any experiments to demonstrate the presence of significant levels of prolactin receptors in our granulosa cells, high levels of specific prolactin receptors

have been previously reported in porcine granulosa cell cultures with 555 binding sites/cell for cells from immature follicles and 300 sites/cell for cells from mature follicles (Rolland and Hammond, 1975). Both immature and mature porcine granulosa cells bound prolactin with an equal affinity of $K_d=0.76$ nM (Veldhuis et al., 1980). Therefore, we feel that our results demonstrate specificity for growth hormone.

The amplified effect of GH on IGF-I-induced progesterone accumulation reported herein could not be attributed to growth or differentiation of granulosa cells. Since the effects were also seen when the amount of progesterone accumulation in cultured granulosa cells was expressed over the amount of cellular protein, DNA content or cell number (Table 1). Neither GH nor IGF-I, or their combination, showed significant differences in plating efficiency or viability of granulosa cells cultured for 96 h (Table 2).

The effects of IGF-I in the ovary have been characterized with great detail. IGF-I promotes both growth (Baranao and Hammond, 1985) and differentiation (Veldhuis et al., 1985) of cultured porcine granulosa cells. IGF-I is able to stimulate progesterone production in granulosa cells at multiple sites in the steroidogenic pathway. These include synthesis of immunoprecipitable cytochrome P450_{scc} enzyme (Veldhuis et al., 1986b) and its corresponding mRNA (Urban and Veldhuis, 1992).

Northern blot analysis indicated that GH amplified IGF-I induced expression of the gene encoding porcine cytochrome P450_{scc} enzyme. This result could be interpreted to suggest that GH-amplified IGF-I induction of progesterone accumulation is mediated, at least in part, by an increase in expression of the gene encoding cytochrome P450_{scc} enzyme.

Recent studies (Samaras et al., 1990) suggest that GH induces the formation of IGF-binding protein-3 (IGFBP-3). IGFBP-3 has been reported to greatly increase the circulating half-life of IGF-I (Cohen and Nissley, 1976) and regulate bioavailability and bioactivity of the IGFs (Gargosky et al., 1994). Due to a pending study of the effect of GH on IGFBPs and IGF-I receptors, our studies do not rule out the possibility that GH mediates its amplified effect on IGF-I action via its effects on IGF-I receptor or IGFBPs.

In conclusion, the results of our present study suggest that GH, in cultured porcine granulosa cells, amplifies IGF-I induced progesterone accumulation in a dose- and time-dependent manner. We postulate that GH sensitizes granulosa cells to IGF-I, and amplifies IGF-I induced progesterone accumulation by a stimulation of the cytochrome P450_{scc} gene expression.

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