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Growth hormone induction of rat granulosa cell tissue-plasminogen activator expression and progesterone synthesis

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Summary

The plasminogen activator (PA) system is present in the ovary and appears to be involved both in follicular growth and ovulation. Similarly, the growth hormone (GH) has been demonstrated to positively affect some ovarian activities. Interestingly, GH appears not only as a mediator of gonadotropin effects, but also as having an independent action of its own on the ovary. In the present study we wanted to investigate if GH could affect ovarian plasminogen activator (PA) activity and steroidogenesis. Granulosa cells from immature rats, injected with pregnant mare serum gonadotropin (PMSG) for inducing follicular growth, were cultured for 24 h with increasing concentrations of GH. A significant dose-dependent increase in tPA activity was observed in the GH-treated cells. This effect was exerted at the mRNA level and the use of cycloheximide, a protein synthesis inhibitor, suggested that GH did not require any other intermediary protein for inducing tPA-mRNA. Furthermore, cAMP levels were not affected by GH treatment. Finally, GH was found to increase progesterone (P) synthesis by granulosa cells. The correlation between the PA system and ovulation and the importance of a normal steroidogenesis for the ovarian physiology claim for a key role of GH in the ovarian activities.

Introduction

The growth hormone (GH) has been clearly demonstrated to be involved in the regulation of several ovarian activities, although its exact role is still controversial. GH is believed to modulate the effects of pituitary gonadotropins on ovarian follicular growth and maturation (Fauser et al., 1988). In rat, GH enhances FSH-induced activities such as the formation of LH/hCG receptors and steroidogenesis from in vitro granulosa cells (Jia et al., 1986; Hutchinson et al., 1988) while in vivo it increases ovarian and renal tissue immunoreactive insulin-like growth factor I (IGF-I) (Davoren and Hsueh, 1986). In cultured porcine granulosa cells GH was shown to enhance the effect of E_2 and FSH on P production (Hsu and Hammond, 1987). Conversely, an effect of GH, by itself, on ovarian steroidogenesis has been recently demonstrated in human. In fact, in granulosa cells GH has a stimulatory effect, independent from that of FSH, on E_2 production (Mason et al., 1990) whereas in luteal cells GH induced P production in a dose-dependent manner and interacted with hCG in a synergistic way (Lanzone et al., 1992). Furthermore, in patients relatively resistant to gonadotropin treatment, concurrent provision of GH reduces the amount of gonadotropin required to induce ovulation (Homburg et al., 1988; Homburg et al., 1990). These evidences strongly indicate an involvement of GH in the regulation of ovarian functions through multiple effects.

The plasminogen activator (PA) system is a general proteolytic system involved in many biological processes such as spermatogenesis, embryo implantation, fibrinolysis, angiogenesis, inflammation and tumor metastasis (Reich, 1978; Dano et al., 1985; Blasi et al., 1987; Saksela and Rifkin, 1988). Two forms of PA exist in mammals: the tissue-type (tPA) and the urokinase-type (uPA) (Dano et al., 1985). Both enzymes cleave plasminogen to form the active protease plasmin. Sev-

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eral lines of evidence suggest that the PA system plays an important role in gonadotropin-induced ovulation (Lipner, 1988). Both types of enzymes are synthesized in the rat ovary, with granulosa cells producing predominantly tPA and thecal cells primarily uPA (Canipari and Strickland, 1985; Ny et al., 1985; Reich et al., 1985); their production is regulated by gonadotropins (Beers et al., 1975; Ny et al., 1985; Reich et al., 1985; Canipari and Strickland, 1986). Furthermore, in the rat gonadotropin-induced ovulation is prevented by injection of inhibitors of serine proteases in the periovarian bursa (Reich et al., 1985; Tsafriri et al., 1989) indicating a role for PA in the ovulatory process.

Based on the demonstration of the ovary as a site of GH action, we undertook the present study to investigate the possible role of GH upon the regulation of PA production and steroidogenesis in cultured granulosa cells from immature PMSG-treated rats.

Materials and methods

Materials

Rats of the Sprague-Dawley strain were obtained form Charles River (Italy). Ovine FSH (oFSH-17) and ovine GH (oGH-15) were obtained from the National Hormone and Pituitary Program of the NIH. The purity of the GH preparation was > 95% and, more specifically, the prolactin (Prl) contamination was less than 1%. Media and reagents were purchased from Sigma Chemical (St. Louis, MO) and Flow Laboratories (Irvine, Scotland). Tissue culture plastics were from Falcon (Becton Dickinson Labware, Lincoln Park, NJ). Carnation nonfat dry milk was from Carnation (Los Angeles, CA). The chromogenic plasmin substrate D-val-leu-lys-p-nitroanilide. 2HCl (S2251) was obtained from Bachem Feinchemikalien AG (Basel, Switzerland). E_2 and P radioimmunologic assay (RIA) kits were obtained from Radim (Pomezia, Italy). Hybond nylon membrane (Amersham) was purchased from Amity (Milan, Italy). cAMP was measured using a RIA Kit purchased from DuPont (Dreieich, Germany).

Preparation of granulosa cultures

Immature (26 day old) female rats were injected subcutaneously with 7 IU of PMSG in 0.1 ml of saline solution. The animals were killed 48 h later. Treatment of immature rats with PMSG induces growth and differentiation of granulosa cells leading to the development of a normal complement of preovulatory follicles (Rao et al., 1991). Granulosa cell cultures were prepared as previously described (Canipari and Strickland, 1985). Briefly, the contents of individual follicles were expressed into medium and the granulosa cells were collected and cultured at 37°C in a 5% CO_2 atmosphere for 24 h. The cells were cultured at a density of $1.5 \times 10^5/200$ μl of McCoy's 5a medium supplemented with 0.1% bovine serum albumin (BSA) and 1 g/l of glutamine.

Assay for plasminogen activator

Enzymatic activity of tPA was assessed by the method of Shimada et al. (1981). Samples were incubated with plasminogen and the plasmin generated was assayed using a chromogenic substrate assay (Verheijn et al., 1982; Andrade-Gordon and Strickland, 1986). In this assay the absorbance generated at 405 nm is related to PA activity. PA activity was expressed in terms of IU/ml with references to a standard preparation of uPA.

Gel electrophoresis and zymography

For zymography of PA, culture fluids were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels under non-reducing conditions (SDS-PAGE) (Laemmli, 1970). Molecular weights were calculated from the position of pre-stained molecular weights markers subjected to electrophoresis in parallel lines. PA was then visualized by placing the Triton X-100-washed gel on a casein-agar-plasminogen underlay as previously described (Granelli-Piperno and Reich, 1978).

RNA preparation and Northern blot

Total RNA was prepared from granulosa cells by the method of Schibler et al. (1980) with some modifications (Huarte et al., 1987). RNAs were denaturated with formaldehyde, electrophoresed on a 1.2% agarose gel containing 6% formaldehyde and transferred to a nylon membrane (Thomas, 1980). Filters were pre-hybridized with hybridization buffer (6 × SSPE, 2.5 × Denhardt's, 0.1% SDS, 0.1 mg/ml salmon sperm DNA, 50% formamide) for 4–24 h at 58°C, then hybridized for 24 h at the same temperature in fresh hybridization buffer (50 μl/cm^2) containing a murine tPA antisense probe. The probe was transcribed from the pSP64-MT_3 plasmid containing the 726-bp *Pvu*II-*Spe*I fragment of the mouse tPA cDNA clone puC9-A33 (Rickles et al., 1988) using SP6 RNA polymerase in the presence of 12.5 μM a ^{32}P-labeled GTP (800 Ci/mmol, NEN Dupont). After hybridization the filters were washed twice at 58°C in 3 × SSC and 0.5% SDS, and once at 75°C in 0.2 × SSC and then exposed to Amersham films at −80°C between intensifying screens for 1–4 days. The filters were then probed with a random primed cDNA for the mouse 18S ribosomal RNA (Arnheim and Kuehn, 1979) after having been stripped with 0.1 × SSC, 0.5% SDS at 95°C for 30 min. Autoradiographs were analyzed with an LKB (Ultroscan XL) laser densitometer to normalize for the relative contents of RNAs in different samples.

Assay for cAMP

At the end of incubation, media were collected, boiled for 15 min and centrifuged at 3000 rpm at 4°C. Supernatants were decanted and stored at −20°C until assayed for cAMP by radioimmunoassay (RIA) (Steiner et al., 1972). Samples were acetylated before the assay following the procedure of Harper and Brooker (1975).

Assay for progesterone and estradiol

The concentration of P and E_2 were determined after 24 h of culture by RIA. The intra-assay and inter-assay coefficients of variation were 4% and 10% respectively for P, and 4% and 5% respectively for E_2.

Statistical analysis

Statistical differences between groups were analyzed by unpaired Student's *t*-test and a *P* value less than 0.05 was considered significant.

Results

Effect of GH on PA activity in cultured rat granulosa cells

To examine the influence of GH on granulosa cell PA production, cells obtained from immature PMSG-treated rats were cultured for 24 h in the presence of medium alone (C), FSH (100 ng/ml) or increasing concentrations of GH (from 0.3 to 1000 ng/ml). At the end of incubation, conditioned media were analyzed for PA activity by enzymatic assay. The addition of FSH to the cells caused an 8-fold induction of PA activity when compared to C. In a manner similar to that of FSH, GH was able to induce PA production by granulosa cells. The induction was dose-dependent with a fold-increase ranging from 0.8 to 5.9 when compared to C and it started to be significant at a GH concentration of 30 ng/ml ($P < 0.001$) (Fig. 1). The two hormones acted synergistically only at suboptimal concentrations whereas at higher GH doses similar levels of PA activity were observed either in the presence or in the absence of FSH (Fig. 2).

In order to characterize the type of PA induced by GH, conditioned media were analyzed for PA activity by SDS-PAGE followed by visualization of the enzyme by putting the gel on a casein-agar-plasminogen film. As reported before, rat granulosa cells secrete predominantly tPA in response to FSH (Canipari and Strickland, 1985; Reich et al., 1985). As with FSH, GH-treatment induced a dose-dependent production of tissue-type PA (Fig. 3).

Time-dependent stimulation of PA secretion by FSH and GH

Granulosa cells were cultured in medium alone (C), or with saturating concentrations of FSH (100 ng/ml) or GH (100 ng/ml) for 2, 4, 8, 12 and 24 h. In control cultures there was only a minimal increase in PA activity over the 24 h-culture period, whereas both GH- and FSH-treated cells showed a time-dependent increase in PA secretion (Fig. 4).

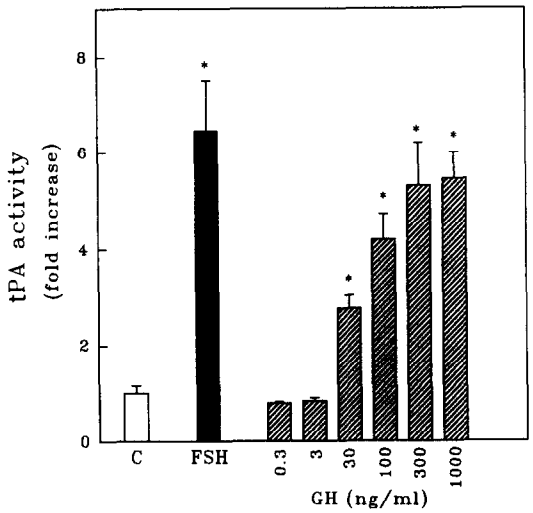

Fig. 1. Dose-dependent stimulation of PA secretion by granulosa cells treated with GH. Cells were cultured in medium alone (C), FSH (100 ng/ml) or increasing doses of GH (0.3–1000 ng/ml). After 24 h, conditioned medium was collected and analyzed for PA activity by chromogenic substrate assay. Results represent the mean ± SEM of eight separate experiments each done in duplicate. Values are expressed as fold induction respect to untreated cells (C = 1.0) * Significantly different ($P < 0.001$) from control cultures.

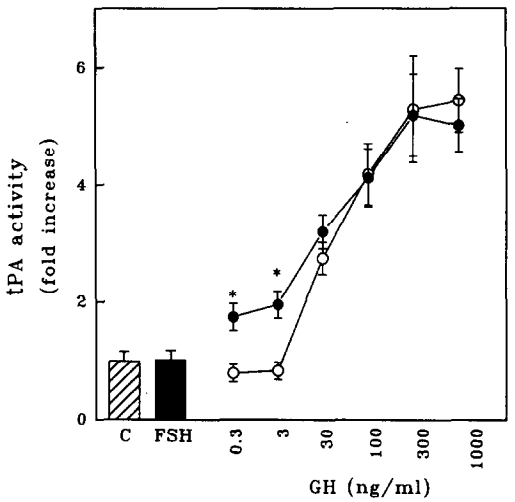

Fig. 2. Effect of increasing concentration of GH on FSH-stimulated PA production in cultured granulosa cells. Cells were cultured in medium alone (C), with FSH (0.5 ng/ml) or with increasing doses of GH (0.3–1000 ng/ml) with (•) and without (○) FSH (0.5 ng/ml). Media were collected and analyzed as described in Fig. 1. Results represent the mean ± SEM of three separate experiments each done in duplicate. Values are expressed as fold induction respect to untreated cells (C = 1.0). * Significantly different ($P < 0.05$) from untreated, FSH- and GH (0.3, 3 ng/ml) treated cultures.

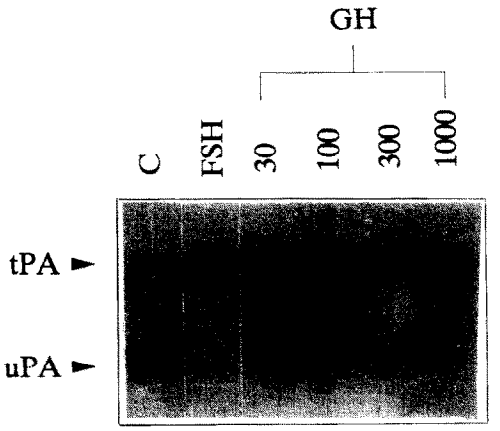

Fig. 3. Zymography of PA secreted by granulosa cells in response to FSH and GH. Rat granulosa cells were cultured in medium alone (C), with FSH (100 ng/ml) or with increasing concentrations of GH (30–1000 ng/ml). After 24 h, conditioned media were collected and analyzed by casein-agar underlay.

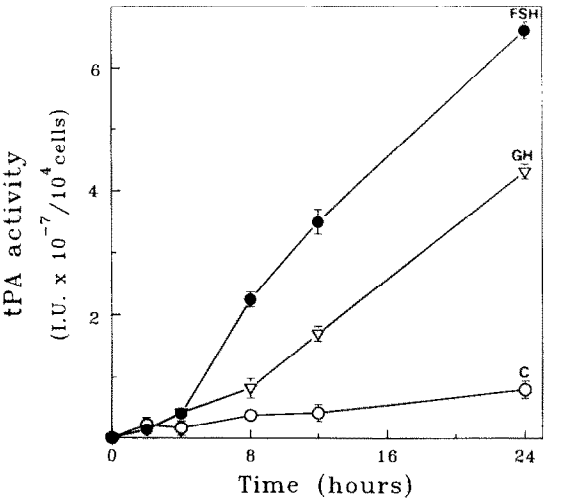

Fig. 4. Time course of PA production by granulosa cells in response to FSH and GH. Granulosa cells were cultured in medium alone (○), 100 ng/ml FSH (●) or 100 ng/ml GH (▽). At the indicated times conditioned media were collected and assayed for PA activity. Results are the mean ± SEM of three separate experiments each done in duplicate.

Effect of FSH, GH and cycloheximide on the steady state levels of tPA mRNA

To determine if GH stimulates tPA-mRNA level, total RNA was extracted from granulosa cells that were cultured for 12 h in medium alone (C), with FSH (100 ng/ml) or with GH (100 ng/ml) and analyzed by Northern blot. The filters were hybridized with a specific murine tPA cRNA probe. Consistent with earlier reports, stimulation of tPA-mRNA by FSH was present (O'Connell et al., 1987). GH too was able to induce tPA-mRNA. In the presence of FSH or GH we observed an increase of the levels of tPA-mRNA of 6- and 4-fold respectively when compared to C (Fig. 5). Thus, these results correlate well with the values of PA activity obtained by enzymatic assay.

In order to investigate if protein synthesis was required for the induction of tPA-mRNA by GH, the protein synthesis inhibitor, cycloheximide, was used. Granulosa cells were cultured with cycloheximide (50 μg/ml) alone or combined with GH (100 ng/ml). Cells were collected 12 h later and RNA was extracted and

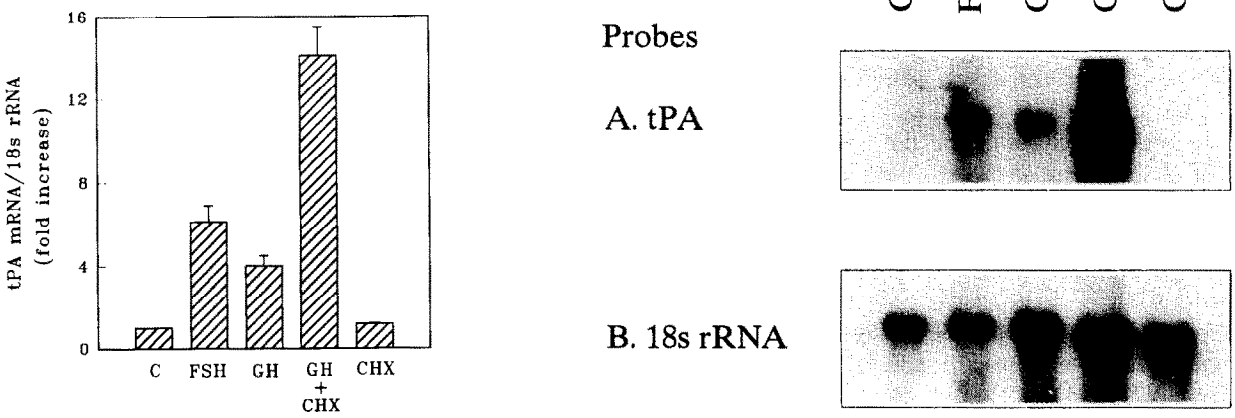

Fig. 5. Northern blot analysis of tPA mRNA levels in granulosa cells treated with GH, FSH and cycloheximide. Cells were cultured for 12 h in medium alone (C), FSH (100 ng/ml), GH (100 ng/ml) with and without cycloheximide (50 μg/ml). Upper panel shows a representative Northern blot. Total RNA (30 μg) was subjected to electrophoresis on a denaturing agarose gel, transferred to a nylon membrane followed by hybridization with a murine tPA cRNA probe (A). The filter was then probed a second time with a random primed mouse 18S ribosomal RNA probe to estimate the amount of total RNA in each lane (B). Lower panel, northern blots were analyzed by densitometer scanning. The amount of tPA mRNA in each lane was normalized for the corresponding 18S rRNA. The normalized values represent the mean ± SEM of two separate experiments and are expressed as fold induction compared to untreated cells (C 1.0).

TABLE 1

EFFECT OF TREATMENT WITH FSH, GH, AND IBMX ON cAMP PRODUCTION BY RAT GRANULOSA CELLS

	cAMP (pmol/1.5×10^5 cells)	
	-IBMX	+IBMX (300 μM)
Control	1.9± 0.9	4.9± 1.7
FSH (100 ng/ml)	108.8±15.7 *	152.3±13.9 *
GH (100 ng/ml)	4.9± 0.9	8.2± 1.1

Granulosa cells were cultured for 5 h in the presence of the different hormones. At the end of incubation extracellular cAMP was measured by RIA. Values represent the mean±SEM of three separate cultures.

* Significantly different ($P < 0.001$) from corresponding control cultures.

analyzed by Northern blot. At the concentration used, cycloheximide caused an inhibition of protein synthesis greater than 95%, therefore no PA activity was detected by zymography (data not shown). As shown in Fig. 5, cycloheximide alone had no effect on the steady-state level of tPA-mRNA whereas it enhanced the tPA-mRNA stimulated by GH.

Effect of GH on granulosa cell cAMP production

To assess whether PA stimulation by GH was dependent on accumulation of cAMP, granulosa cells were cultured for 5 h with medium alone (C), FSH (100 ng/ml) or GH (100 ng/ml) in the presence or absence of the phosphodiesterase inhibitor IBMX (300 μM). As expected FSH significantly increased the extracellular level of cAMP and the addition of IBMX further enhanced this effect. On the contrary, GH with or without IBMX did not greatly affect cAMP levels as well as IBMX alone (Table 1).

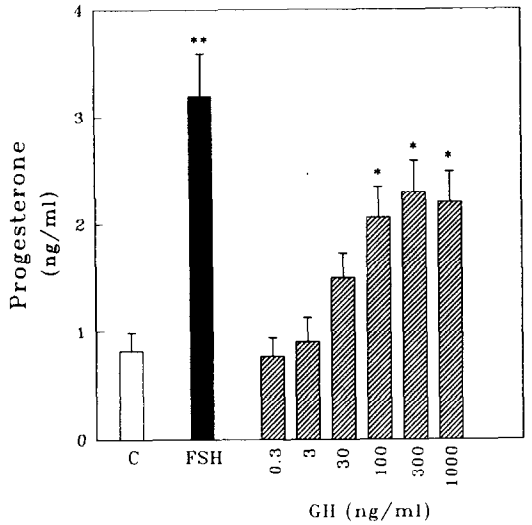

Fig. 6. Effect of FSH and GH on progesterone produced by granulosa cells. Cells were cultured in medium alone (C), with FSH (100 ng/ml) or with increasing concentration of GH (0.3–1000 ng/ml). After 24 h, conditioned media were collected and assayed for P. Results represent the mean±SEM of three separate experiments each done in duplicate. ** $P < 0.005$, * $P < 0.05$ compared to control cultures.

Effect of GH on granulosa cell steroidogenesis

The possible effect of GH on P and E_2 production was evaluated in granulosa cells that were cultured for 24 h in medium alone (C), with FSH (100 ng/ml), or with increasing concentrations of GH (from 0.3 to 1000 ng/ml). As shown in Fig. 6, GH induced a dose-dependent increase in P production and a plateau was reached at 100 ng/ml. The GH-stimulated P levels were lower that those obtained with FSH but not significantly different. In control cultures high levels of E_2 were detected, therefore no further effect of GH or FSH on E_2 production was observed.

Discussion

In the present study we provide the first evidence that GH, by itself, is capable of increasing production of tPA and P by granulosa cells from immature PMSG-treated rats. These effects contrast with previous results where GH alone dit not affect tPA activity but synergized the gonadotropin positive effect on such enzymatic activity (Jia et al., 1986; Hsu and Hammond, 1987; Hutchinson et al., 1988). One explanation for the difference between those results and ours could be the fact that for this study we utilized granulosa cells from immature PMSG-primed rats instead of granulosa cells from immature hypophysectomized animals. In fact, the granulosa cells we used are, compared to the others, much more differentiated with a content of LH receptors and a steroidogenic capacity similar to that of mature cycling rats (Rao et al., 1991). Therefore, GH could have different effects depending on the stage of maturation of the follicle.

Also, it is known that GH can react with Prl receptors and then its effect on ovarian tPA activity could be mediated through lactogenic receptors. However, a recent study demonstrated that Prl inhibits PA activity in preovulatory follicles (Yoshimura et al., 1990) suggesting that GH may stimulate tPA activity through its own receptors.

We found GH to induce, in vitro, tPA activity in a dose- and time-dependent manner. The action of GH on PA production was statistically significant at 30 ng/ml, a concentration that is within the physiological range (Ojeda and Jameson, 1977; Terry et al., 1982). The latter consideration, i.e. the fact that low concentrations of GH are sufficient to significantly induce PA production and the purity of the GH preparation used for our experiments, should further rule out the possibility that the GH effects are due to gonadotropin or Prl contamination. Moreover, the fact that the values of tPA activity obtained after GH stimulation were not

significantly different from those obtained with FSH supports the physiological relevance of the present in vitro study.

In previous studies on rat granulosa cells GH has been shown to act as a gonadotropin modulator (Jia et al., 1986; Hsu and Hammond, 1987; Hutchinson et al., 1988). The present data show that the combined action of the two hormones was synergistic only at low concentrations, whereas this effect was not detectable at higher doses at which probably the PA system is already maximally stimulated.

The time course of tPA activity induced by GH was different from that of FSH suggesting an alternate mechanism of action for GH. It is well known that granulosa cell tPA activity is stimulated by gonadotropins (Canipari and Strickland, 1985; Ny et al., 1985; Reich et al., 1985), GnRH (Ny et al., 1987; Canipari et al., 1989), epidermal growth factor (EGF) (Galway et al., 1989), and basic fibroblast growth factor (bFGF) (LaPolt et al., 1990), all hormones that presumably act through different second-messenger systems. In rat granulosa cells GH treatment increases FSH-stimulated cAMP production (Jia et al., 1986). Therefore, the action of GH on granulosa cell functions may be partially mediated through increase in cAMP production. However, the non-significant augment in the cAMP levels observed in the granulosa cell cultures at the end of GH treatment suggests that the action of GH on these cells was probably not mediated through cAMP and protein kinase A pathway.

The exact mechanism by which GH exerts its effects on the ovary is still controversial. GH may interact with its receptors and directly accomplish its effects. Also, GH may stimulate ovarian production of IGFs which in turn mediate GH effects. Supporting this possibility is the observation that in vivo GH treatment increases the ovarian content of immunoreactive IGF-I in the rat (Davoren and Hsueh, 1986) and in vitro GH stimulates the production of IGF-I by porcine granulosa cells (Hsu and Hammond, 1987). We observed that the action of GH in inducing tPA activity was exerted at the steady-state level of tPA-mRNA. This effect was a direct effect of GH on the cells. In fact, in the presence of cycloheximide, a protein synthesis inhibitor, the GH effect was fully expressed and actually amplified suggesting that at least in this case, GH does not require neither IGFs nor other intermediary protein synthesis. The superinduction of tPA mRNA observed in the presence of GH and cycloheximide was similar to that seen in FSH-stimulated granulosa cells (Ohlsson et al., 1988).

Based on previous studies, a possible role of GH on ovarian steroidogenesis was suggested (Jia et al., 1986; Hsu and Hammond, 1987; Mason et al., 1990; Lanzone et al., 1992). Our results show that as for tPA activity GH stimulated P synthesis in a dose-dependent manner. Furthermore, P levels induced by GH were not significantly different from those stimulated by FSH. This effect was a specific effect of GH on granulosa cells since it was independent from gonadotropin. At the present we have no elements to discriminate if this action was or was not mediated by other molecules such as IGFs. The lack of effect on E_2 observed in this study can be attributed to the already high levels of estrogen production in PMSG-treated rats in which the PMSG treatment maximally induced granulosa cell aromatase (Rao et al., 1991).

In conclusion, our findings together with those already described for rodents and human strongly suggest an involvement of GH in the ovarian activities. What remains to be answered are the exact mechanisms and eventually the other factors by which GH mediates its local effects in the ovary.

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