Endocrine, *vol. 6, no. 2, 145–151, April 1997* 0969–711X/97/6:145–151/$9.75

Regulation of Ovarian Follicle Differentiation in Gonadotrophin-Stimulated Rats

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The aim of the present study was to investigate the regulation of the in vitro DNA synthesis of ovarian cells recovered from prepubertal rats 48 h after administration of pregnant mare's serum gonadotrophin alone (granulosa cells) or followed by human chorionic gonadotrophin (luteal cells). Isolated granulosa cells were cultured in serum-free medium, different stimuli added for periods of 48 h, and ^{3}H-thymidine incorporation was measured. Both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) inhibited ^{3}H-thymidine incorporation by cultured granulosa cells in a dose-dependent manner (FSH: 10, 100, 200 ng/mL = 26, 41, 49% inhibition, respectively; LH: 0.1, 1, 10 ng/mL = 11, 37, 75% inhibition, respectively). On the other hand, estradiol was found to stimulate ^{3}H-thymidine incorporation in granulosa cells (Estradiol: 5, 50, 500 ng/mL = 17, 37, 76% stimulation, respectively). In luteal cells, the rate of basal ^{3}H-thymidine incorporation was very low (granulosa cells: 2560 ± 310; luteal cells: 661 ± 92 cpm/100,000 cells) and not modified by any stimulus. To determine the possible production of an inhibitory growth factor by the early corpus luteum, ^{3}H-thymidine incorporation by granulosa cells was assessed in the presence of 10% conditioned media (CM) recovered from luteal cell cultures. A marked inhibition both in basal and estradiol-stimulated ^{3}H-thymidine incorporation was observed (74 and 76% of inhibition, respectively). Results suggest that an inhibitory growth factor produced by luteal cells after luteinizing gonadotrophin stimulus could be involved in the differentiation of growing follicles to corpus luteum.

Key Words: Follicle differentiation; ovary; corpus luteum; granulosa cell.

Introduction

The follicular phase of the ovarian cycle is characterized by the rapid proliferation of follicle cells, whereas its postovulatory stage is distinguished by the differentiation of granulosa cells to form the corpus luteum, in a process termed luteinization.

It has been accepted that the main function of the corpus luteum is the production of progesterone, which prepares the uterus for embryo implantation and the maintenance of the first period of pregnancy. Both processes require the integrity of the corpus luteum. If fertilization and implantation do not occur, the ovarian cycle is reinitiated only after corpus luteum function ceases. In addition, previous to the development of the corpus luteum, granulosa cell proliferation must be inhibited *(1,2)*. Whereas follicle-stimulating hormone (FSH), estrogens *(3–5)*, and growth factors *(6–14)* are responsible for ovarian follicle proliferation, there is little information available regarding the factors involved in the inhibition of mitotic activity following the luteinizing hormone (LH) surge, nor on the regression of the corpus luteum *(15,16)*. It has been demonstrated that hCG inhibits DNA synthesis in bovine corpus luteum and rat granulosa cells *(3)*, and that the mitotic activity of human granulosa cells is arrested in response to the LH surge *(17)*. In addition, an intrinsic self-regulating mechanism has been postulated. In this regard, the laboratory has described the presence of a cell-growth inhibitory factor in the conditioned media from human granulosa cell cultures *(18)*.

The aim of the present study was thus to determine: the effects of different stimuli on the incorporation of thymidine to DNA of rat follicular cells, and the possible production by the early corpus luteum of a factor regulating the growth of granulosa cells.

Results

Effect of PMSG Treatment on Ovarian Parameters

Subcutaneous injection of immature, prepubertal rats with 25 IU of pregnant mare's serum gonadotrophin (PMSG, Novormon) was associated with an increase in weight (control: 12.1 ± 1.7; PMSG: 30.9 ± 3.7 mg; $p < 0.05$) and size (control: 3.75 ± 0.26; PMSG: 5.24 ± 0.34 mm; $p < 0.05$) of the ovaries and in the number of viable granulosa cells recovered per ovary (control: 483,700 ± 82,300; PMSG: 2,061,000 ± 87,500; $p < 0.05$). The latter effect was corroborated by the determination of protein content/ovary (control: 142.8 ± 17.3; PMSG: 735.1 ± 48.3 μg; $p < 0.05$).

Received September 30, 1996; Revised January 2, 1997; Accepted January 2, 1997.
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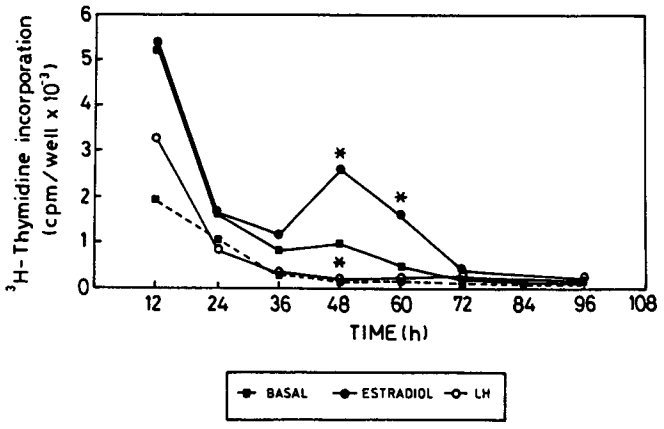

Fig. 1. Time-course of in vitro thymidine incorporation by granulosa cells obtained from nontreated (control) or PMSG-treated rats. 75,000 cells/well were seeded in 96-well culture plastic dishes precoated with collagen. hLH (10 ng/mL) or estradiol (500 ng/mL) were added at t (time) = 0 h. ^{3}H-thymidine (0.4 μCi/well) was added at t = 0, 12, 24, 36, 48, 60, 72, or 84 h; in all cases, cultures were harvested 12 h later. Solid lines: PMSG-treated rats. Dashed lines: nontreated rats (control). Values are expressed as the mean ± SEM of quadruplicate cultures. Asterisks indicate significant differences ($p < 0.05$) by analysis of variance (ANOVA) and Scheffé's multiple range test.

Time-Course of Thymidine Incorporation in Granulosa Cells

Thymidine incorporation was chosen as a measure of DNA synthesis instead of the usual approaches of measuring cell numbers. This technique is useful and more sensitive because of the low in vitro replicating potential of rat granulosa cells since it was demonstrated that only a small cell fraction is able to enter into the S phase *(9,10)*.

To test whether the stimulatory or inhibitory effects of certain stimuli on granulosa cell DNA synthesis were time-related, thymidine incorporation by these cells was measured during 12-h consecutive culture periods (Fig. 1). Estradiol and LH were chosen as stimuli, because of their demonstrated receptor content in mature granulosa cells *(19)*. The point indicated as t = 0 h corresponds to the moment of addition of the stimuli. Different wells were treated with either 500 ng/mL estradiol or 10 ng/mL LH, and media were not changed for the duration of the experiment. Tritiated thymidine was added at t = 0, 12, 24, 36, 48, 60, 72, and 84 h. In all cases, cultures were harvested 12 h later as indicated. As shown in Fig. 1, granulosa cells obtained from nontreated prepubertal rats showed comparatively low levels of thymidine incorporation during the entire culture period. Estradiol, caused a slight, nonsignificant stimulation at t = 72 h, as described by Bendell et al. *(4)*, whereas LH had no effect (data not shown).

In contrast, granulosa cells obtained from PMSG-treated prepubertal rats showed a high rate of T incorporation during the initial 48 h following plating. In these cultures, the higher thymidine incorporation levels seen at recovery time ($p < 0.05$, t = 12) probably reflect the completion of DNA synthesis induced in vivo by PMSG treatment *(20)*. After 24 h of culture, thymidine incorporation declines, likely because of disruption during isolation of the gap junctions between granulosa cells and because of the fact that cultures are performed under serum-free conditions. In spite of some disadvantages, a defined medium was selected because the presence of gonadotrophins, steroids, and other factors as yet poorly defined in serum, limits the interpretation of the results obtained. As shown in Fig. 1, in the presence of 500 ng/mL estradiol, thymidine incorporation increased at 48–60 h and then returned to basal levels, whereas in cells cultured in the presence of 10 ng/mL LH, thymidine incorporation was lower than basal wells at 12–60 h (Fig. 1). This latter phenomenon could not be interpreted as a toxic, nonspecific effect of LH on the cultures, since this hormone does not affect plating efficiency or in vitro tritiated thymidine incorporation in granulosa cells obtained from control rats. Thus, this low thymidine incorporation could be attributed to an in vitro LH stimulation of the luteinization process.

In accordance with these results, all subsequent experiments were performed adding ^{3}H-thymidine 24 h after plating followed by harvesting after an additional 24 h.

Effects of Different Stimuli on Thymidine Incorporation in Granulosa Cells

In vitro thymidine incorporation by granulosa cells obtained from PMSG-treated rats could be modified (increased or diminished) by the addition of certain stimuli. Figure 2 shows the dose-response curves of gonadotrophins on thymidine incorporation. Both FSH and LH were found to be inhibitory. The lower inhibitory doses were: 10 ng/mL and 1 ng/mL respectively, presumably reflecting a physiological phenomenon. Accordingly, dibutyryl cyclic adenosine monophosphate (cAMP) could mimic this effect on granulosa cells at a 0.1 m*M* dose (data not shown). On the other hand, prolactin only showed inhi-bitory behavior at extremely high doses (1 μg/mL), prob-ably a result of a contamination of the prolactin sample with LH.

Fig. 2. *(opposite page)* Dose-response curves of different adenohypophyseal hormones on granulosa cell ^{3}H-thymidine incorporation. Granulosa cells were isolated from PMSG-treated rats and cultured 48 h in the presence of increasing doses of oFSH, hLH, or prolactin. Thymidine was added 24 h after plating and cells were harvested 24 h later. Values are the mean ± SEM of quadruplicate wells. Values with the same superscript are not significantly different ($p > 0.05$) by ANOVA and Scheffé's multiple range test.

Fig. 3. *(opposite page)* Dose-response curves of different steroid hormones on granulosa cell ^{3}H-thymidine incorporation. Granulosa cells were isolated from PMSG-treated rats and cultured 48 h in the presence of increasing doses of estradiol, progesterone, testosterone or dihydrotestosterone (DHT). Thymidine was added 24 h after plating and cells were harvested 24 h later. Data are mean ± SEM of quadruplicate wells. Values with the same superscript are not significantly different ($p > 0.05$) by ANOVA and Scheffé's multiple range test.

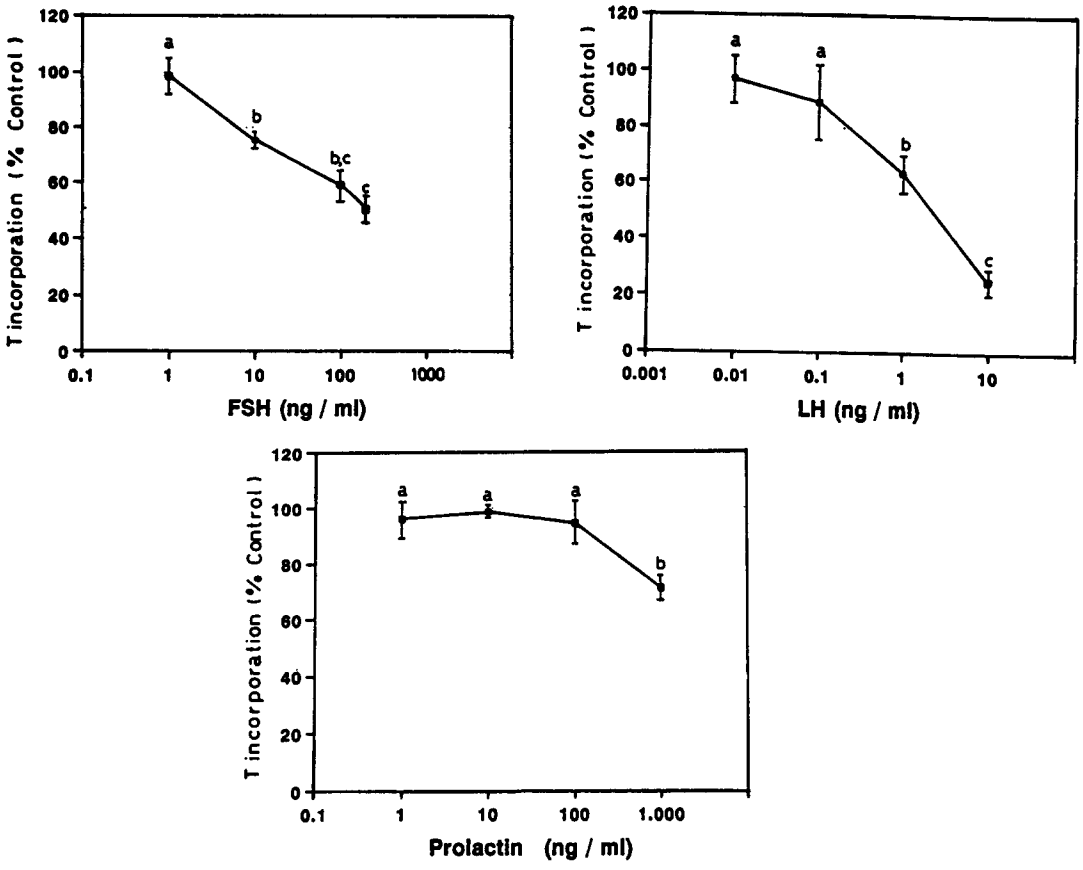
T incorporation (% Control)
FSH (ng / ml)
LH (ng / ml)
Prolactin (ng / ml)

Fig. 2.

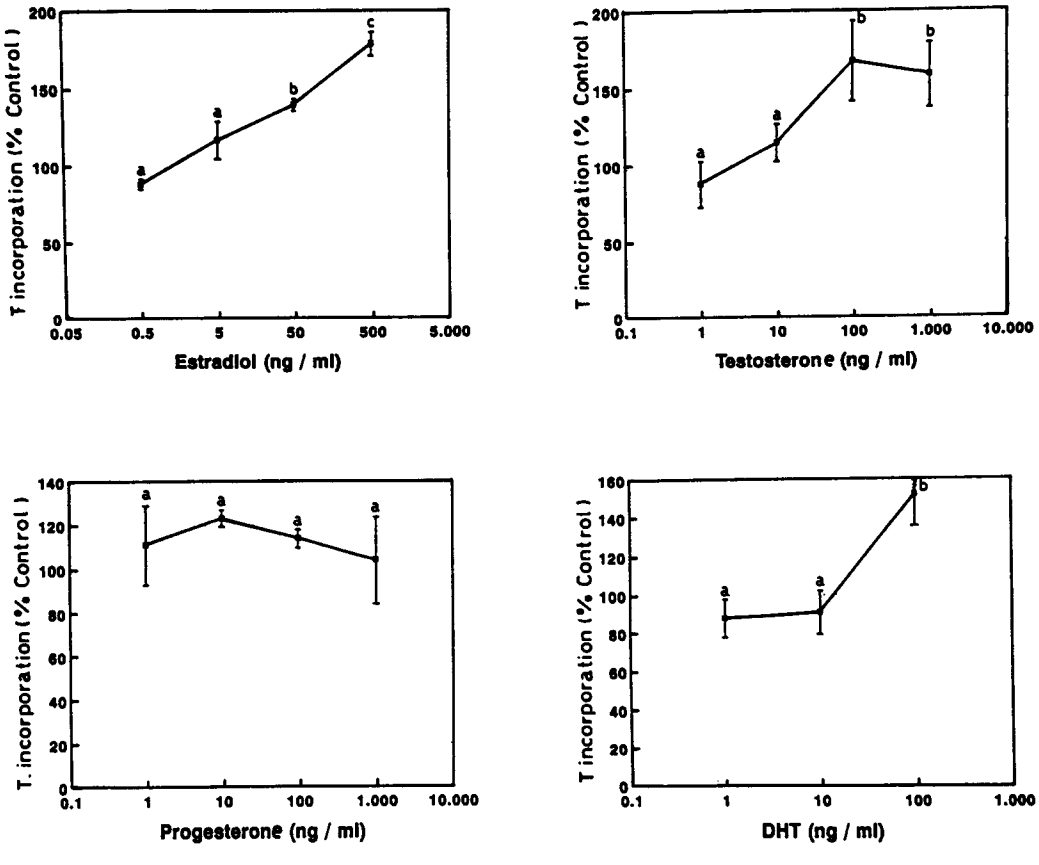
T incorporation (% Control)
Estradiol (ng / ml)
Testosterone (ng / ml)
T. incorporation (% Control)
Progesterone (ng / ml)
T incorporation (% Control)
DHT (ng / ml)

Fig. 3.

Table 1
Effect of Different Stimuli on ^{3}H-thymidine Incorporation in Ovarian Cells from [PMSG + hCG]-Treated Rats

Treatment	cpm/well
—	561 ± 98
hLH (10 ng/mL)	220 ± 36
oFSH (200 ng/mL)	368 ± 65
Prolactin (1 μg/mL)	374 ± 41
Estradiol (500 ng/mL)	402 ± 56
Progesterone (100 ng/mL)	484 ± 80
Testosterone (100 ng/mL)	440 ± 36
DHT (100 ng/mL)	403 ± 48

Ovarian cells were obtained from rats treated with PMSG and hCG. 100,000 cells/well were seeded on collagen-coated 96-well plastic dishes and cultured 48 h in the presence of the different stimuli. Thymidine was added 24 h after plating and cells were harvested 24 h later. (Blanks values = 92 ± 8 cpm/well). Results are the means ± SEM of quadruplicate wells.

The effects of different steroid hormones on thymidine incorporation are shown in Fig. 3. 17β-estradiol, testosterone, and a nonaromatizable androgen (DHT) produced a significant stimulation of T uptake at a concentration range of 50–500 ng/mL. Nevertheless, these results could not be attributed to a generalized steroid hormone action, since progesterone did not produce this effect at similar doses (Fig. 3).

Effect of In Vivo PMSG+hCG Treatment on Thymidine Incorporation in Ovarian Cells

The subcutaneous administration of hCG (25 IU) to prepubertal rats treated 48 h earlier with PMSG caused a significant reduction of ^{3}H-thymidine incorporation in ovarian cells (PMSG: 2560 ± 310; PMSG + hCG: 661 ± 92 cpm/well, $p < 0.05$). In this case, the massive ovulation and subsequent luteinization of mature follicles mediated by hCG *(21)* could account for this reduction. At recovery time (48 h after hCG injection), the majority of ovarian cells had a postmitotic granulosa–lutein or theca–lutein phenotype. The low incorporation rate of these cells could not be reverted in vitro by the addition of any stimuli (Table 1). On the other hand, this low level of thymidine incorporation observed in ovarian cells from PMSG + hCG-treated rats could not be attributed to the different cell isolation method or to its consequence, the mixed ovarian cell populations in the culture, since collagenase-dispersed preparations of ovarian cells obtained from PMSG treated rats were able to incorporate even more thymidine than mechanically-obtained (granulosa cell-enriched) cultures (7269 ± 595 cpm vs 3726 ± 414 cpm; $p < 0.05$). In addition, plating efficiency was not modified by the isolation procedure (around 35%).

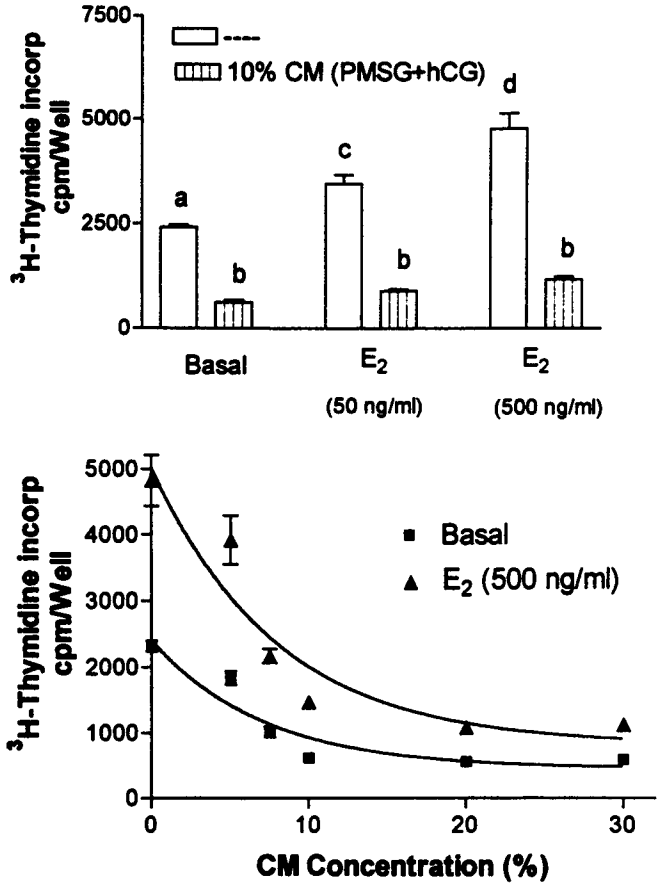

Fig. 4. Upper panel: effect of 10% CM obtained from luteal cell cultures (PMSG + hCG-treated rats) on ^{3}H-thymidine incorporation (24-h pulse) in cultured mature granulosa cells (PMSG-treated rats). Granulosa cells were cultured 48 h with (striped bars) or without (open bars) CM in the absence (Basal) or presence of estradiol (E: 50 or 500 ng/mL). ^{3}H-thymidine was added 24 h after plating, and cultures were harvested 24 h later. Data are the mean ± SEM of quadruplicate wells. Values with the same superscript are not significantly different ($p > 0.05$) by analysis of variance and Scheffé's multiple range test. Lower panel: CM dose response curve obtained in similar conditions.

Effect of Ovarian Luteinized Cell-Conditioned Media on Thymidine Incorporation and Steroid Production in Granulosa Cells

As shown in Fig. 4 (upper panel), the presence of 10% conditioned media (CM) obtained from luteal cell cultures produced a decrease in the thymidine incorporation of granulosa cells. This effect could also be observed in granulosa cell cultures stimulated by estradiol (50 or 500 ng/mL). The lower panel shows the effect of different concentrations of CM on granulosa cell thymidine incorporation. Controls using CM from 48-h granulosa cell cultures did not produce any change (data not shown). These results suggest that luteal cells but not granulosa cells produce a factor capable of inhibiting granulosa cell growth.

On the other hand, the effects of CM addition (10%) to granulosa cell cultures on progesterone and estradiol accumulation were assayed. Estradiol production was measured

in cultures containing androstenedione (0.25 μ*M*) as substrate. CM did not modify the steroidogenic response of granulosa cells (Progesterone = basal: 5.86 ± 1.19; CM: 6.13 ± 0.76; estradiol = basal: 12.15 ± 0.25; CM: 14.15 ± 1.53 ng/mL.) Similar results were obtained with dibutyryl cAMP-stimulated granulosa cells.

Discussion

The regulation of the mechanisms involved in the transformation of mature follicles to corpus luteum is unclear. Although the periovulatory LH surge is mainly responsible for this process, it remains unclear whether the gonadotrophin acts directly or through the production of some ovarian factor/s. The findings of the present study support this last concept by showing that the serum-free CM from luteal cells from rats treated with PMSG-hCG inhibits the DNA synthesis of PMSG-treated rat granulosa cell cultures. Moreover, thymidine incorporation observed in these cultured luteal cells shows a significant decrease in relation to that obtained using mature granulosa cell cultures. These results would demonstrate that an autocrine or paracrine inhibitory growth factor is produced by ovarian follicles after in vivo treatment with hCG. However, since the luteal cell preparations were not purified, the possibility of an effect from endothelial cells or macrophages on the results obtained cannot be discarded. Previous studies demonstrated that rat luteinized follicles do not incorporate tritiated thymidine *(22)* and that after exposure to LH the mitotic index falls and there is an increase in cytoplasmic volume *(23)*. Yong et al. *(13)* show the opposite effect for FSH and LH in human granulosa cells: whereas FSH enhances in vitro DNA synthesis, LH suppresses it. The disparity in the action of both gonadotrophins seems to be related to the substantially higher cAMP levels elicited by LH. High doses of cAMP are found to be inhibitory, whereas low doses are stimulatory for DNA synthesis *(24)*. In our case, the addition of LH to the culture medium produces a decrease in mature granulosa cell proliferation measured as ^{3}H-thymidine incorporation. Unexpectedly, in this system FSH produces the same effect. A possible explanation could be the previous increase in adenylate cyclase activity elicited after in vivo exposure to PMSG that could later be amplified by the in vitro FSH stimulus, giving rise to high cAMP levels similar to those obtained by LH stimulation. However, the possibility cannot be discarded that these mature, more differentiated granulosa cells, have a different response to that described for immature granulosa cells.

Previous studies have suggested that estrogens can act directly on rat ovaries to stimulate the proliferation of ovarian cells *(25–27)*. The presence of specific binding sites for estradiol has been demonstrated in rat granulosa cells *(19,28)*, as well as androgen receptors *(29,30)*. Thus, following interaction with their specific binding sites, the ability of these steroids to elicit an increase in thymidine incorporation in granulosa cell cultures might be related to a direct action of the hormone on cellular proliferative capacity or mediated by the production of growth factors. In this regard, Bendell and Dorrington *(4)* have demonstrated that estradiol stimulates the production of transforming growth factor-β by theca cells and that this factor mediates the mitogenic effect of estradiol on rat granulosa cells. In the experimental model, some theca cell contamination in the granulosa cell preparation could be responsible for the results described. Interestingly, both testosterone and DHT, a nonaromatizable androgen, increase the thymidine incorporation in granulosa cell cultures, suggesting that the effect is mediated by binding to its own receptors and not because of conversion to estrogens. On the other hand, the fact that progesterone did not produce any effect on thymidine incorporation in granulosa cells discards the possibility of a nonspecific action of the steroid.

The stimulating effect of estradiol on granulosa cell DNA synthesis is also inhibited by the addition of serum-free CM from luteal cell cultures. However, the effect would be specific on the proliferative process since the addition of CM on granulosa cell cultures did not modify steroid production. These results are in agreement with previous studies *(18)* showing the antiproliferative effect of conditioned medium from human granulosa-lutein cells on immature rat granulosa cell cultures stimulated by FSH and estradiol. In these experiments, granulosa cells were obtained from DES-treated rats, a model in which the in vitro addition of FSH is necessary to attain cell proliferation. Thus, it is probable that FSH is not a requirement to stimulate DNA synthesis in our cultures since animals were previously treated with PMSG. The inhibitory growth factor produced by human luteinized granulosa cells is a protein of a molecular weight >30,000 Daltons *(18)*. Further studies will be necessary in order to characterize the factor produced by rat luteal cells in culture and to compare it with this former protein.

In summary, it is concluded that the DNA synthesis of granulosa cells obtained from PMSG-treated rats was inhibited by gonadotrophins and stimulated by estradiol and androgens. Treatment of PMSG-injected rats with hCG specifically decreased the thymidine incorporation of ovarian cells. This effect could not be reverted by any of the stimuli used in this study. CM from luteal cells obtained from PMSG-hCG injected rats inhibited the thymidine incorporation of granulosa cell cultures. It is proposed that the existence of an ovarian cell factor/s would inhibit the proliferation of granulosa cells. This factor could be involved in the differentiation of the mature follicle to corpus luteum.

Materials and Methods

Materials

PMSG was kindly donated by Syntex S.A. (Buenos Aires, Argentina). Human chorionic gonadotrophin (hCG, Endocorion) was obtained from Elea Laboratories (Buenos

Aires, Argentina). Ovine FSH (oFSH S17), human LH (hLH: 7.2 IU/μg), and ovine prolactin (AFP-8277E), were kindly provided by The National Hormone and Pituitary Agency, NIADKK-NIH (Bethesda, MD). According to the specifications of NIADDK, the LH activity of ovine prolactin measured by radioimmunoassay was <0.5% by weight. The absence of LH bioactivity of the oFSH NIH-S17 preparation was demonstrated by the inability of oFSH to stimulate in vitro testosterone production by isolated Leydig cells (unpublished data). Steroid hormones, HEPES, EGTA, EDTA, deoxyribonuclease type I (DNase I), trypsin, dibutyryl cAMP, and bovine serum albumin (fraction V, bovine serum albumine, BSA) were from Sigma Chemical Co. (St. Louis, MO). Collagenase type CLS II (125 IU/mg) was obtained from Worthington Biochemical Co. (Freehold, NJ). Dulbecco's Modified Eagle Medium (4.5 g glucose/L), Ham F-12 nutrient mixture, Medium 199 with Earle's salts, fungizone (250 μg/mL) and gentamicine (10 mg/mL) were obtained from Gibco Laboratories (Grand Island, NY). All other chemicals were of reagent grade from standard commercial sources.

Animals and Experimental Procedures

Female Sprague-Dawley rats (23–25-d-old), were kept under controlled environmental conditions (22°C, lights-on from 0700 to 1900 h) and had free access to pellets rat chow and tap water. Rats were nontreated (control) or treated with 25 IU PMSG 48 h earlier, to induce follicular maturation. PMSG-treated rat ovaries were not luteinized at the moment of the isolation since this treatment does not cause an endogenous luteinizing hormone surge until 52–54 h after hormone administration *(31)*. Each group of ovaries was weighed and measured. A group of rats treated with PMSG was injected 48 h later with 25 IU of hCG and sacrificed 48 h after hCG administration. This treatment results in superovulation and a considerable increase in ovarian weight because of the stimulation and subsequent transformation of ovarian follicles into corpus luteum *(32)*.

The experimental protocols were approved by the Animal Experimentation Committee of IBYME.

Granulosa Cell Isolation

Granulosa cells from control and PMSG-treated rats were isolated according to Bley et al. *(10)*. Briefly, the ovaries were punctured with a sterile needle and incubated 15 min at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g glucose/L): Ham F-12 nutrient mixture (1:1), 10 m*M* HEPES, 50 μg/mL gentamicin, and 250 ng/mL fungizone (DMEM-HEPES), containing 6.8 m*M* EGTA. Ovaries were then washed twice and incubated 5 min in DMEM-HEPES with 0.5*M* sucrose. Following the incubation, the medium was diluted with DMEM-HEPES and the ovaries were allowed to sediment. Granulosa cells were obtained by gently pressing the ovaries between two pieces of nylon mesh. In order to obtain purified granulosa cell cultures, the cell suspension was layered onto a 44% Percoll cushion and centrifuged 20 min at 400*g*. Cell number, as well as protein contents were determined both for control and PMSG-treated groups.

Isolation of Ovarian Luteinized Cells

Ovaries obtained from PMSG+hCG-treated rats were minced and incubated 40 min at 37°C in DMEM-HEPES containing 1 mg/mL collagenase, 0.008% DNase I and 0.5% BSA. After incubation, the collagenase-dispersed tissue was washed twice and luteal cells were obtained by gently pressing the suspension between two pieces of nylon mesh.

Ovarian Cell Culture

Ovarian cells were centrifuged 10 min at 250*g*, the supernatants discarded, and the pellets resuspended in serum-free Medium 199 with 2.2 g/L $NaHCO_3$ for counting in a hemocytometer. Viability, assessed by Trypan Blue exclusion, ranged from 30–40% in granulosa cell preparations and from 55–65% in luteinized cells. Cells were then seeded on 96-well plastic culture plates precoated with rat-tail collagen *(33)*. The initial plating density was 100,000 cells/well in a final volume of 0.1 mL. Cells were maintained in a humidified incubator for 2 h, after which media were changed to remove nonattached cells and stimuli or luteal conditioned medium added. Optimum stimulus doses were previously determined. In order to calculate plating efficiency, 16 h after change of media, cultured cells were trypsinized and counted. Granulosa and luteinized cell plating efficiency was approx 35% and was not modified by any of the stimuli assayed.

Thymidine Incorporation Assay

Tritiated thymidine (0.4 μCi/well, final specific activity 1.2 Ci/mmol; Dupont, Boston, MA) was added to the cultures 24 h after stimuli or luteal conditioned medium addition. After a further 24 h cells were harvested in hollow glass fibers using a multiwell harvester *(18)*. Excess ^{3}H-thymidine was removed by washing with 6 vol of distilled water followed by one of ethanol. Filters were allowed to dry at least 12 h before being transferred to vials and radioactivity counted in a scintillation counter (efficiency 50%).

Recovery of Ovarian Luteinized Cell Conditioned Media

To obtain luteal CM, luteinized cells were cultured as described above, CM removed 48 h after seeding and centrifuged 10 min at 250*g*, after which pellets were discarded and supernatants stored at –20°C until their assay on granulosa cell cultures. CM from 48-h granulosa cell cultures treated in the same manner was employed as control.

Other Methods

Progesterone and estradiol production by granulosa cells was evaluated by RIA in suitable dilutions of the culture media. RIAs were performed as described previously *(34)*, using specific antibodies supplied by Dr. G. D.

Niswender, CO. In the present conditions, the within-assay and between-assay variations were 8.0 and 14.2, respectively for progesterone; and 7.2 and 12.5, respectively for estradiol.

Protein content in granulosa cells was determined according to Lowry et al. *(35)*, after dissolving the samples for 16 h in 0.5 NaOH at 20°C. Bovine serum albumin was employed as standard.

Statistical Analysis

Results are expressed as means ±SEM of quadruplicate culture dishes. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Scheffe's multiple range test. Probability values <0.05 were considered significant. Each experiment was repeated at least three times using cells from different groups (6–8 rats per group). The illustrated experimental results were obtained from representative experiments using quadruplicate culture wells.

Acknowledgments

The present study was funded by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (0488/88-National Research Council, Buenos Aires, Argentina), Roemmers Foundation (Buenos Aires, Argentina), and the Universidad de Buenos Aires (UBA EX-149-Buenos Aires, Argentina).

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