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6. The method of S. Umezawa and H. Watanabe [*J. Exp. Biol.* **58**, 305 (1973)] has been used in a modified version. Velocity of water flow through the respiratory tube was regulated by adjusting the height of the supply tank. Flow rate has been determined by collecting water from the outlet of the tube at the beginning and end of a series of experiments. The larva is stabilized with a small piece of cotton in the tube that does not interfere with the free movements of the pectoral fin. The percentage of oxygen in the water before and after passage through the respiratory tube was determined with an oxygen analyzer (Beckman). Within a 50-minute period, the larva remained quiet. Data were obtained at 10-minute intervals from 8 to 12 sets of readings. At this stage the larva does not swim, eliminating the possibility that the difference between countercurrent and concurrent measurements are due to swimming efforts.
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Growth Factors Modulate Gonadotropin Receptor Induction in Granulosa Cell Cultures

Abstract. *Epidermal growth factor and fibroblast growth factor inhibited follicle-stimulating hormone-dependent induction of luteinizing hormone receptor in cultured ovarian granulosa cells of the rat. In contrast, platelet-derived growth factor potentiated the induction of luteinizing hormone receptor by follicle-stimulating hormone. These results indicate that growth factors, well known for their effects on mitosis and DNA synthesis in cultured mammalian cells, are also able to modulate hormone-dependent differentiation in an endocrine target cell.*

Induction of receptor for luteinizing hormone (LH) is a critical aspect of granulosa cell differentiation and ovarian follicular development, because it is by this process that the follicle acquires responsiveness to LH and the capacity to ovulate and luteinize. Follicle-stimulating hormone (FSH) was established as an inducer of granulosa cell LH receptor in vivo (*1*) and the effect has now been documented in vitro under chemically defined culture conditions (*2*). In addition to their role as recipients of hormonal stimuli, granulosa cells of a number of species (rabbit, pig, human, and guinea pig, but not rat) are known targets for the mitogenic and growth-promoting actions of growth factors in vitro (*3*). The nature of the relation between growth factors and more highly differentiated granulosa cell functions has only begun to be appreciated (*4*). We report here that growth factors can modulate FSH-dependent granulosa cell LH receptor induction in vitro (*5*) and propose that such compounds may represent a physiologically relevant control system interacting with gonadotropic and other endocrine components to regulate ovarian cellular differentiation.

Granulosa cells from immature, diethylstilbestrol-primed rats were cultured for 3 days in the absence of FSH or growth factors to allow monolayers to establish. They were then cultured for another 3 days with or without purified human FSH, with or without growth factors, or with both FSH and growth factors. At the end of the 6 days, LH receptor binding was assessed by the specific binding of ^{125}I-labeled human chorionic gonadotropin (hCG) (*6*).

The effect of epidermal growth factor (EGF) on FSH-dependent LH receptor induction is shown in Fig. 1. In granulosa cells cultured in serum-free, chemically defined medium in the absence of FSH, LH receptor binding was comparable to that in cells before culture (data not shown). Treatment with FSH during the last 3 days of culture increased LH receptor binding nearly 15-fold ($P < .001$). Epidermal growth factor alone did not suppress LH receptor binding but it blocked the FSH-induced increase. The effect was dose-related with respect to both EGF and FSH. EGF completely inhibited LH receptor induction at 10 ng/ml but only partially inhibited it at 1 ng/ml. The inhibitory effect of EGF at 1 ng/ml was more pronounced at higher doses of FSH. Such a trend suggests that EGF may act, at least in part, to enhance any down-regulatory action of FSH on the newly induced LH receptor (*7*). The EGF did not compete with ^{125}I-labeled hCG in the binding assay (data not shown) indicating that the inhibitory action of EGF was exerted on receptor induction during the culture period.

Suppression of FSH-dependent LH receptor induction occurred in the absence of a pronounced growth-promoting or mitogenic effect of EGF, as assessed by the yield of cell protein after culture (*8*). This observation is in agreement with the reported resistance of rat granulosa cells to growth factor–stimulated mitogenesis (*3*).

The effect of EGF on progestin secretion was also investigated. The amount of 20α-dihydroprogesterone (20α-OH-P) in spent media from cells cultured with or without human FSH or growth factors during culture days 3 to 6 was determined by radioimmunoassay (*9*). We found that EGF (10 ng/ml) did not impair FSH-stimulated (250 ng/ml) progestin secretion: when expressed as micrograms of 20α-OH-P per milligram of cell protein per 3 days (mean ± standard error; $N = 3$) the basal level was 0.59 ± 0.01; with EGF the result was 0.49 ± 0.07; with FSH, 4.89 ± 0.19; and with FSH

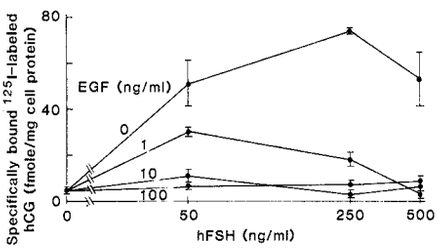

Fig. 1. The effect of EGF on FSH-dependent LH receptor induction. The results show the relation between dose and response for EGF and FSH. Ovarian granulosa cells were isolated from estrogen-treated immature female rats and incubated at 37°C in a humidified atmosphere of 5 percent CO_2 in air in multiwell plates (Falcon). The wells were first treated with human fibronectin (2 to 4 μg/cm^2, Collaborative Research, Waltham, Mass.) to facilitate cell attachment. Each well contained approximately 10^6 cells and 1.0 ml of culture medium consisting of McCoy's 5A supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml, Gibco) and insulin (25 mIU/ml, Eli Lilly, porcine sodium insulin, lot No. 050YB7). After 3 days the cultures received fresh 5A, antibiotics, insulin, and human follicle-stimulating hormone (*hFSH*) and EGF where indicated. After 6 days, specific binding of ^{125}I-labeled hCG was assessed. Total binding was determined in triplicate cultures with 75 ng of ^{125}I-labeled hCG in a final volume of 250 μl of culture medium containing 10 percent fetal calf serum. Duplicate cultures were incubated as described above following 30 minutes in the presence of a 250-fold excess of unlabeled hCG (APL, Ayerst Laboratories) to determine the nonspecific binding for each treatment group. After 2 hours at 37°C the binding reaction was terminated by washing with cold phosphate-buffered saline (PBS). Cells were scraped from the culture wells with a rubber policeman into 1 ml of cold PBS and centrifuged from the buffer. Radiologic counts were normalized on the basis of cell protein (protein assay kit No. 1, Bio-Rad Laboratories). The amount of labeled hCG bound was calculated from the specific activity of the labeled hCG preparation and a molecular weight of 47,500. Specific binding was taken to be the difference between total and nonspecific binding. Data points represent means ± standard error, $N = 3$.

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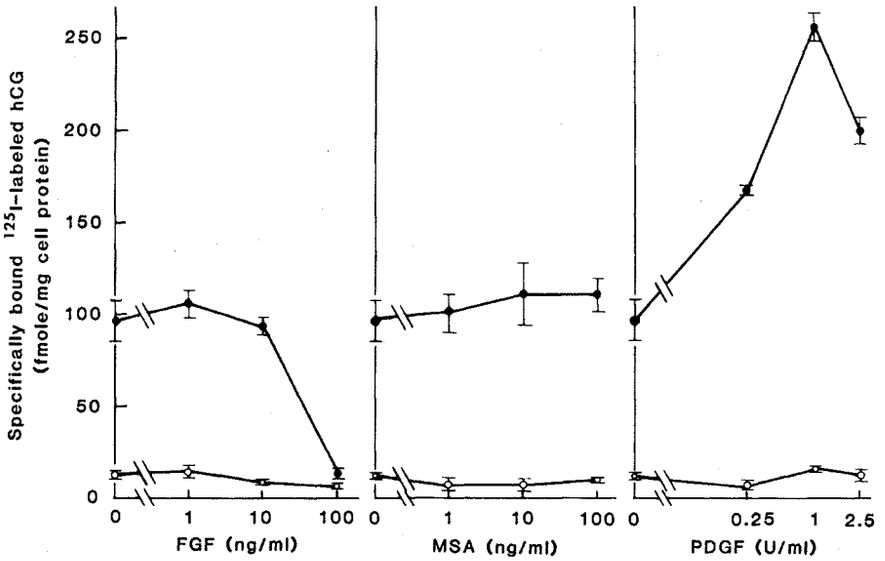

Fig. 2. Effects of FGF, MSA, and PDGF on FSH-dependent LH receptor induction. Methods were as described in the legend for Fig. 1. Symbols: ○, without FSH; ●, with hFSH (250 ng/ml). Data points represent means ± standard error, $N = 3$.

plus EGF, 5.71 ± 0.40. This suggests that the inhibition of FSH-dependent LH receptor induction by EGF did not result from a total lack of cellular responsiveness to FSH.

To determine whether EGF was acting at the level of the LH receptor itself, granulosa cell monolayers were treated with FSH (250 ng/ml, on culture days 3 to 6) to induce LH receptor binding (specifically bound ^{125}I-labeled hCG, 39.5 ± 3.3 fmole per milligram of cell protein, $N = 3$) which was then monitored with time in the presence and absence of EGF (10 ng/ml). After the FSH was removed at day 6, LH receptor binding determined on days 7 and 8 revealed a decline (culture day 7, 30.1 ± 1.4; day 8, 18.8 ± 0.8). In the presence of EGF, the decline was more pronounced (day 7, 33.6 ± 2.5; day 8, 6.9 ± 3.3, $P < .05$, compared to day 8 in the absence of EGF), indicating that EGF may act, in part, to accelerate the loss of LH receptor once formed. The mechanism of such an effect is unclear. EGF is known to be rapidly internalized after it becomes bound to its receptor (*10*). The possibility that unoccupied LH receptor is co-internalized with EGF receptor is unlikely since the time-course of LH receptor loss observed in the presence of EGF was much longer than would be consistent with rates reported for EGF receptor loss in granulosa cells (*11*).

Other growth factors were tested and found to vary in their effects on receptor induction (Fig. 2). Fibroblast growth factor (FGF) inhibited LH receptor induction by FSH similarly to EGF, whereas platelet-derived growth factor (PDGF) potentiated the action of FSH. Multiplication-stimulating activity (MSA) had no effect over the dose-range tested, and none of the growth factors had any effect on LH receptor binding in the absence of FSH (*12*).

Scatchard analysis of FSH-induced LH receptor binding indicated the presence of a single class of high-affinity binding sites. Potentiation of LH receptor binding following treatment with FSH plus PDGF was characterized by an increase in binding capacity with no change in affinity. Similarly, the attenuation of binding noted after treatment with FSH plus EGF was due to a change in capacity only (*13*).

We have demonstrated that FSH-dependent LH receptor induction, a critical aspect of follicle development, is acutely sensitive to both positive and negative modulation by growth factors in vitro. These observations suggest a possible regulatory role for growth factors in vivo. EGF-like inhibitors that attenuate LH receptor binding activity may be involved in the failure of follicle development which characterizes follicular atresia. The facilitatory activity of PDGF may have implications for LH receptor induction during luteal cell development if PDGF or similar factors are released during follicle rupture or formation of the corpus hemorrhagicum. Growth factors may thus represent physiological effectors that have been heretofore unrecognized among the more classical determinants of ovarian cellular development. In addition, the study of growth factor-gonadotropin interactions in granulosa cell cultures may lead to the development of a useful, biochemically defined model system with which to investigate broader aspects of endocrine-dependent differentiation at the cellular level.

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6. Highly purified human FSH (hFSH, LER-1577) was kindly provided by L. E. Reichert, Jr. Growth factors were obtained from Collaborative Research (Waltham, Mass.) and used without further purification. Highly purified hCG, kindly provided by D. Puett, was labeled with radioactive iodine using lactoperoxidase [R. L. Stouffer, L. Tyrey, D. W. Schomberg, *Endocrinology* **99**, 516 (1976)] to specific activities of 32.5 ± 5.4 μCi/μg (mean ± standard deviation). Maximum binding capacity of the labeled material (45 to 50 percent) was determined by binding to excess LH receptor of porcine granulosa cells.
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8. Over the dose-range of EGF tested, 1 to 100 ng/ml, cultures yielded only 1.0- to 1.6-fold more protein than those which did not receive EGF, whether or not FSH was present; no obvious dose-response correlation could be made. This small increment is probably due, at least in part, to variations in cell seeding densities among cultures, and is not sufficient to explain the lower LH receptor binding associated with EGF as a protein dilution phenomenon.
9. Antibody to 20α-dihydroprogesterone-3-*o*-carboxymethyloxime bovine serum albumin was obtained from Steranti Research Ltd., England. In agreement with results of other workers, greater than 95 percent of the total progestin content of spent culture media samples was 20α-OH-P [A. Nimrod, *Mol. Cell. Endocrinol.* **8**, 189 (1977)].
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12. The FGF, MSA, and PDGF had no effect on FSH-stimulated 20α-OH-P secretion, suggesting no effect on FSH responsiveness. Growth-promoting or mitogenic effects were not evident for these growth factors.
13. Scatchard analysis, which was based on the specific binding of ^{125}I-labeled hCG in the presence of various doses of tracer, revealed an apparent dissociation constant (K_d) of 1.9×10^{-10} M. Functionality of FSH-induced (with or without growth factors) LH receptor sites was assessed by the steroidogenic response to hCG (20α-OH-P secretion). Results were difficult to interpret; cultures washed of FSH continued to secrete large amounts of progestin and no hCG-dependent increment could be demonstrated.
14. We thank A. Brown and S. Bass for technical assistance. This research formed a portion of a dissertation submitted by J.S.M. to Duke University in partial fulfillment of the requirements for the Ph.D. Supported in part by grant HD-11827 from the NICHHD, USPHS.
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