

Note

Modulatory Role of Epidermal Growth Factor in Follicle-Stimulating Hormone-Induced DNA Synthesis in Cultured Rat Granulosa Cells

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Abstract. Epidermal growth factor (EGF) modestly increased DNA synthesis by cultured rat granulosa cells. FSH also stimulated DNA synthesis dose-relatedly with the maximal effect occurring at FSH 100 ng/ml. The stimulatory effect of FSH was still greater than that of EGF. However, in the presence of EGF, the stimulatory effect of FSH at any concentration was regulated to the level as high as when EGF alone stimulates. In addition, EGF inhibited DNA synthesis induced by forskolin, but enhanced the action of (Bu)₂cAMP additively, which indicates that EGF attenuates DNA synthesis of granulosa cells by suppressing the activity of adenylate cyclase or cAMP production. As it is suggested that cAMP is the most likely intracellular second messenger for FSH, the regulatory effect of EGF on FSH-induced DNA synthesis could be, in part, due to suppression of cAMP production. These results suggest that EGF is involved in granulosa cell proliferation, irrespective of the presence of FSH, in a different pathway from FSH and additionally modulates the FSH growth-promoting effect as a local regulator. The interaction between EGF and FSH may be important in the control of follicular development.

Key words: Granulosa cell, EGF, FSH, DNA synthesis.

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THIS IS INCREASING evidence that growth factors serve as intraovarian modulatory factors in follicular development. Among various polypeptide growth factors, epidermal growth factor (EGF) is detected in porcine [1] and human follicular fluid [2] and shown to be synthesized by thecal/interstitial cells [3]. In addition, granulosa cells possess the receptor for EGF in several species [4–7]. These findings provide strong support for a physiological role of EGF in the development of the follicle. Indeed, a large number of reports in the literature thus far indicate that EGF regulates various functions of granulosa cells.

No doubt gonadotropins play a central role in

the proliferation and the differentiation of granulosa cells. Recent studies have demonstrated the modulatory action of EGF on FSH-induced differentiated functions of granulosa cells; for example, that EGF inhibits FSH-mediated induction of LH/hCG receptors [8] and attenuated FSH-mediated estrogen production [9]. Since EGF itself acts as a mitogen in granulosa cells, it is of interest to determine whether it may modulate the mitogenic effect of FSH which is a key tropic hormone in follicular development.

Materials and Methods

Materials

The materials were obtained from the following sources: pregnant mare serum gonadotropin (PMSG) from Teikoku Hormone MFG. Co., Ltd.

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(Tokyo, Japan), insulin, penicillin G and porcine follicle-stimulating hormone (FSH) from Sigma Chemical Company (St. Louis, USA), estradiol, progesterone and testosterone from MERCK ART. (Germany), mouse EGF (receptor grade) from Collaborative Research, Inc. (Lexington, USA), methyl- ^3H -thymidine (specific activity 2.9 MBq/mmol) from NEN Research Products (Boston, USA), forskolin from Behring Diagnostics Inc. (La Jolla, USA), $(\text{Bu})_2\text{cAMP}$ from Boehringer Mannheim GmbH (Germany) and Medium 199 from GIBCO (Grand Island, USA).

Granulosa cell culture

Immature 25-day-old female Wistar rats were treated with 10 IU PMSG in 0.2 ml saline by sc. injection. Forty-eight h after the injection, the ovaries were removed and granulosa cells were collected free of oocytes as described before [7]. Approximately 5×10^4 cells were seeded into Corning 24-multiwell plates (No. 25820) in a total volume of 0.5 ml Medium 199 supplemented with 2% fetal bovine serum, 5 $\mu\text{g}/\text{ml}$ of insulin and 3.5 $\mu\text{g}/\text{ml}$ of penicillin G and incubated in humidified 95% air and 5% CO_2 at 37°C. The cell viability determined by trypan blue exclusion exceeded 90% in every sample. After 48 h, various specified agents were added to the culture medium and the cell culture was continued for a further 24 h.

^3H -Thymidine incorporation growth assay

On day 3, the cells were cultured in medium containing 1 $\mu\text{Ci}/\text{ml}$ methyl- ^3H -thymidine for 20 h, after which they were washed twice with 1 ml of phosphate-buffered saline on ice. After treating the cells with ice-cold 10% and 5% trichloroacetic acid, they were solubilized in 0.5 ml of 1 N NaOH and the radioactivity was determined in a liquid scintillation counter.

Statistical analysis

All data are presented as the mean \pm SEM of 6 separate determinations. Student's *t*-test was used for the statistical evaluation of the results. Observations were confirmed in two or more independent experiments. A value of $P < 0.05$ was assumed to be statistically significant.

Results

Figure 1 illustrates the effect of EGF on the amount of DNA synthesized by cultured granulosa cells. The addition of EGF increased the amount of thymidine incorporated into DNA in a dose-dependent manner at a range of concentrations between 0.1 and 50 ng/ml. A significant stimulatory effect was observed at concentrations as low as 1 ng/ml. As depicted in Fig. 2, the addition of FSH increased DNA synthesis by cultured rat granulosa cells in a concentration-dependent way between 0.5 and 100 ng/ml. At a concentration of 100 ng/ml, FSH increased DNA synthesis by 150%. The degree of increase in DNA synthesis by FSH was more pronounced than that caused by EGF. However, the coincubation of FSH with EGF resulted quite differently. In the presence of EGF, FSH at any concentration augmented DNA synthesis only up to a level as high as EGF alone stimulated. Even at a concentration of 100 ng/ml, FSH could not induce a marked increase in the DNA synthesis of the granulosa cells treated with EGF.

To gain insight into the mechanism whereby EGF eliminated the stimulatory effect of FSH, we examined whether EGF might modulate the en-

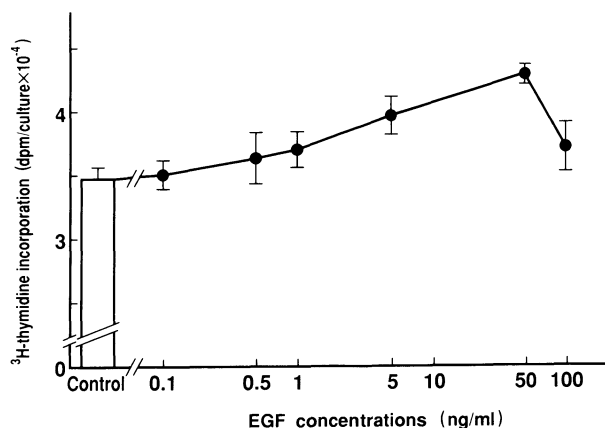


Fig. 1. Stimulatory effect of EGF on ^3H -thymidine incorporation in cultured rat granulosa cells. Granulosa cells were cultured with increasing concentrations (0.1–100 ng/ml) of EGF for 44 h. ^3H -thymidine was added to the culture media for the last 20 h. Incorporation of ^3H -thymidine was determined as described in "Materials and Methods". Each value is the mean \pm SEM of 6 determinations.

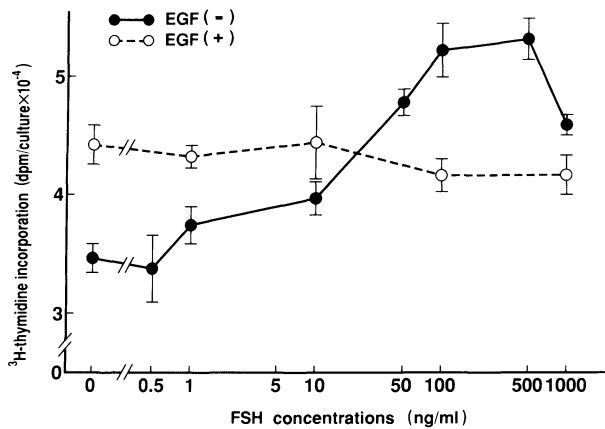


Fig. 2. Interaction between FSH and EGF and effect on ^3H -thymidine incorporation into DNA of cultured rat granulosa cells. Granulosa cells were cultured with increasing concentrations (0.5–1000 ng/ml) of FSH in the absence or presence of EGF (50 ng/ml) as described in Fig. 1. ^3H -thymidine incorporation in cells was determined as described in "Materials and Methods". Each value is the mean \pm SEM of 6 determinations.

hanced DNA synthesis evoked by forskolin, an activator of adenylate cyclase, and $(\text{Bu})_2\text{cAMP}$ (Fig. 3). As expected, these two agents significantly increased DNA synthesis. As with FSH, the stimulatory effect of forskolin on DNA synthesis was completely eliminated by the addition of EGF. In contrast, EGF further augmented the stimulatory effect of $(\text{Bu})_2\text{cAMP}$ additively.

Discussion

Several lines of evidence thus far presented suggest EGF as an intraovarian regulator acting in concert with gonadotropins. Previous studies have shown that EGF stimulates the proliferation of granulosa cells isolated from man [10], cattle [11, 12] and pigs [10, 13, 14]. However, so far no reports have been found documenting the mitogenic effect of EGF in rat. In the present study, granulosa cells were cultured in the medium supplemented with insulin. In the absence of insulin, we could not observe any growth-promoting effect of EGF. Therefore, the reason why EGF exhibited a mitogenic effect in this system may be the coincubation of EGF with insulin. At any rate, the mitogenic effect

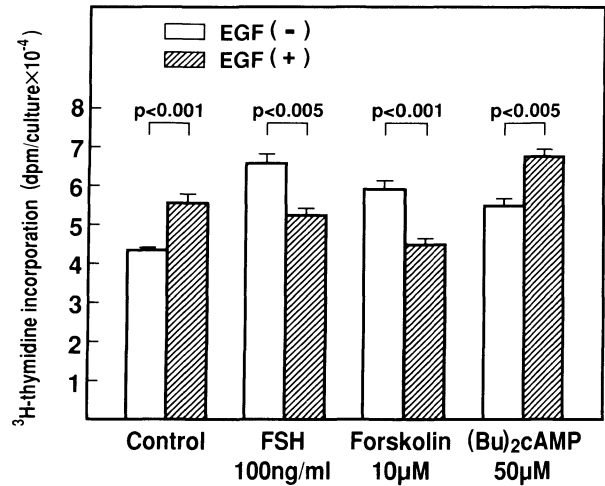


Fig. 3. Effect of treatment with EGF on basal, FSH-, forskolin-, $(\text{Bu})_2\text{cAMP}$ -induced ^3H -thymidine incorporation into the DNA of cultured rat granulosa cells. Granulosa cells were treated with medium alone or medium containing FSH (100 ng/ml), forskolin (10 μM) and $(\text{Bu})_2\text{cAMP}$ (50 μM) in the presence or absence of EGF (50 ng/ml). Each value is the mean \pm SEM of 6 determinations.

of EGF in rat is modest and less prominent than that of FSH.

In this study, EGF is shown to attenuate the stimulatory effect of FSH on the proliferation of granulosa cells without altering its own growth-promoting effect. This finding may have important implications related to a better understanding of the physiological role of EGF in follicular development. A primordial follicle after recruitment undergoes rapid growth, during which time granulosa cells multiply noticeably. However, at the beginning of development it seems that immature follicles grow little influenced by FSH [15, 16]. On the other hand, granulosa cells from immature follicles have EGF receptors with higher binding capacity than those from mature follicles [4]. Therefore, it is conceivable that EGF may play an important role in stimulating the growth of granulosa cells in the early stage of follicular development. In contrast, mature follicles which acquire sufficient amounts of FSH receptors are responsive to FSH and FSH may act as a mitogen as well as an inducer of differentiation. In this connection, it is intriguing to speculate that EGF attenuates FSH-induced cell proliferation, thus preventing excessive growth of the follicle in preparation for ovulation.

An additional example of dual modulation by a local growth factor has been presented. Transforming growth factor β (TGF β) is assumed to be one of the autocrine/paracrine factors in follicular development. This peptide enhances the amount of LH receptors induced by a suboptimal concentration of FSH, whereas it rather suppressed the LH receptor expression in the presence of an optimal concentration of FSH [17]. Thus it appears that growth factors may modulate the actions of gonadotropins in either a synergistic or an antagonistic way depending on the maturational state of granulosa cells. TGF β notably amplifies the stimulatory effect of FSH on EGF receptors [18]. Furthermore, it augments the inhibitory effect of EGF on FSH-induced LH receptors [18]. There seem to be a variety of intraovarian regulators that are in play in the process of follicular development with complex interplay amongst them.

Recent reports suggested that cAMP, which is the most likely intracellular second messenger for FSH, induced not only differentiation but proliferation of granulosa cells in several species [19, 20]. The present study indicated that EGF inhibits forskolin-induced DNA synthesis. A previous study demonstrated that EGF suppresses FSH-induced adenylate cyclase activity [21]. Since the action of FSH is mediated by the generation of cAMP, it is possible that the inhibitory effect of EGF on FSH-stimulated DNA synthesis is, in part, due to the suppression of cAMP production or interference with the action of adenylate cyclase by EGF. In contrast, EGF combined with (Bu)₂cAMP additively increased DNA synthesis, suggesting a

cAMP independent mechanism of EGF action. Consequently, it is possible that the proliferative potency of granulosa cells is determined by the balance between cAMP activity and a cAMP independent intracellular mechanism of EGF.

The previous study demonstrated that EGF enhances FSH-binding to cultured porcine granulosa cells [22]. Conversely, FSH increases EGF binding capacity in rat granulosa cells [6]. These observations suggest a positive interaction between EGF and FSH by potentiating the binding capacity of each other. In keeping with this, EGF augments 3 β -hydroxysteroid dehydrogenase induced by FSH [9]. However, a negative interaction of the two has been also exemplified. For instance, EGF inhibits FSH-induced aromatase activity [23, 24] and LH/hCG binding capacity [8]. Therefore, the mode of interaction between EGF and FSH seems to vary as to the index of differentiation and cannot be explained exclusively on the basis of the receptor status.

There has been growing concern about the physiological roles of local growth factors involved in follicular development. However, at present, many studies are dealing with in vitro systems, observing the actions of growth factors with little attention to physiological relevance. It is apparent that gonadotropins are the primary regulators of follicular development. Our current concern is an understanding of how local regulators modulate the actions of gonadotropins to assure an exquisitely controlled process of follicular maturation. The present study may provide some clues in elucidating the mechanism of follicular growth.

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