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Opposite Effects of Basic Fibroblast Growth Factor on Gonadotropin-Stimulated Steroidogenesis in Rat Granulosa Cells

MAREO YAMOTO, TOSHIHIKO SHIKONE, AND RYOSUKE NAKANO

Department of Obstetrics and Gynecology, Wakayama Medical College, Wakayama 640, Japan

Abstract. In the present study, we examined the effect of basic fibroblast growth factor (bFGF) on gonadotropin-dependent estradiol and progesterone production in cultured rat immature and FSH-primed granulosa cells. Treatment with bFGF alone did not affect the biosynthesis of estradiol and progesterone in immature and FSH-primed granulosa cells. Basic FGF significantly inhibited FSH-induced estradiol and progesterone production in both immature and FSH-primed granulosa cells. In contrast, bFGF exerted a significant stimulatory effect on LH-induced estradiol and progesterone production in FSH-primed granulosa cells. Our finding that bFGF exerts opposite effects on gonadotropin-dependent steroidogenesis suggests that bFGF may play important paracrine and/or autocrine roles in the process of follicular development, ovulation, and subsequent luteinization.

Key words: Granulosa cell, bFGF, Steroidogenesis, FSH, LH. (*Endocrine Journal* **40**: 691–697, 1993)

BASIC FIBROBLAST growth factor (bFGF), a 155-amino acid polypeptide, is a potent mitogen for cells of mesodermal and neuroectodermal origin [1]. A growing body of evidence has suggested a role for bFGF in the regulation of ovarian functions. Recently, we have demonstrated the existence of specific binding sites for bFGF on rat granulosa cells [2]. Adashi *et al.* [3] have shown that bFGF decreases FSH-stimulated estrogen biosynthesis in rat immature granulosa cells. In addition, bFGF is shown to inhibit the FSH-induced LH receptor expression [4, 5] and inhibin subunits expression in cultured rat granulosa cells [6]. Basic FGF therefore appears to function as a potent inhibitor of maturation of immature granulosa cells *in vitro*.

On the other hand, it has been reported that bFGF is capable of increasing granulosa cell tissue-type plasminogen activator activity and message levels, inducing oocyte maturation and increasing follicular prostaglandin (PG) E content [7]. We have reported that FSH induces functional receptors for bFGF in rat granulosa cells [2]. Since the corpus luteum angiogenic factor is related to bFGF [8], the growth factor is thought to exert profound regulatory effects in the process of ovulation and luteinization. Although it is possible that bFGF may play a regulatory role in the luteinized granulosa cell, little is known about the interrelationship between the growth factor and the steroidogenesis in the luteinized granulosa cell. In the present study, we examined the effect of bFGF on gonadotropin-dependent estradiol and progesterone production in both immature and FSH-primed granulosa cells.

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Correspondence to: Dr. Mareo YAMOTO, Department of Obstetrics and Gynecology, Wakayama Medical College, 27 Shichibancho, Wakayama 640, Japan.

Materials and Methods

Hormones and reagents

McCoy's 5a medium (modified; without serum), penicillin-streptomycin solution, L-glutamine and

trypan blue stain (0.4%) were obtained from Grand Island Biochemical Co. (Santa Clara, CA, USA). Ovine FSH (NIH oFSH-S17; FSH activity, 20 × NIH-FSH Sl U/mg, LH activity 0.04 × NIH-LH S1 U/mg), ovine LH (oLH-26.2.3 U/mg, FSH contamination <0.5% by weight) were obtained from the National Hormone and Pituitary Distribution Program, NIDDKD, NIH (Baltimore, MD, USA). Recombinant human bFGF was provided by Synergen (Boulder, CO, USA). Diethylstilbestrol (DES) and androstenedione were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Granulosa cell cultures

Intact immature female Sprague-Dawley rats (25 days old) were obtained from Kiwa Laboratory Animals Co. (Wakayama, Japan) and injected sc DES (1 mg) dissolved in sesame oil daily for 4 days. A 14 h light, 10 h dark cycle was maintained with the light cycle initiated at 0600 h. The animals were sacrificed by cervical dislocation. Granulosa cells were obtained from ovaries by needle punctures as previously described [2]. The cells (1×10^5 viable cells/tube) were cultured in polystyrene tubes (Falcon Plastics, Los Angeles, CA, USA) (12 × 75 mm), containing 0.5 m*l* McCoy's 5a medium (modified; without serum) supplemented with 2 mM L-glutamine, 100 U/m*l* penicillin, 100 μg/m*l* streptomycin sulfate, and 10^{-7} M androstenedione. Cell cultures were performed in a humidified 95% air- 5% CO_2 incubator at 37°C. In some experiments, granulosa cells were cultured for 48 h in the presence or absence of oFSH (30 ng/m*l*) with or without bFGF. In others, the granulosa cells were initially cultured for 2 days in the presence of 30 ng/m*l* oFSH and, after priming, the cells were washed twice with medium and reincubated in fresh culture medium for additional 48 h in the presence or absence of the specified experimental agents. At the end of the experiments, media collected were stored frozen at −80°C until assayed for estradiol or progesterone content by RIA as previously described [9].

Assay of estradiol and progesterone

Estradiol concentrations in the cultured media were determined with a commercial RIA kit (Daiichi Radioisotope Laboratories, Tokyo, Japan) without extraction by diethyl-ether. The antiserum was raised against estradiol-17β-carboxymethyloxime-bovine serum albumin (BSA). This antiserum cross-reacts 0.47% with estrone and 0.41% with estriol but shows no cross-reaction with testosterone, androstenedione, dehydroepiandrosterone, progesterone, 17α-OH-progesterone, 17α-OH-pregnenolone, or cortisol.

Progesterone concentrations in the cultured media were determined with a commercial RIA kit (Daiichi Radioisotope Laboratories, Tokyo, Japan) without extraction by diethyl-ether. The antiserum was raised against progesterone-hemisuccinate-BSA. This antiserum cross-reacts 0.24% with 17α-OH-progesterone, 1.2% with deoxycorticosterone, 6.6% with corticosterone, and 72.9% with 11α-OH-progesterone but shows no cross-reaction with estradiol, cortisol, testosterone, cortisone, pregnenolone, or 11-deoxycortisol. The intra-assay and interassay coefficients of variation for these assay did not exceed 5.1% and 5.6%, respectively.

Data analysis

All experimental data were presented as the mean ± SEM of duplicate measurements of triplicate cultures. Statistical analyses were performed by the analysis of variance with a multiple range test.

Results

The addition of FSH (30 ng/m*l*) to the cells resulted in remarkable increase in the production of both estradiol (Fig. 1A) and progesterone (Fig. 1B). Treatment with bFGF (30 ng/m*l*) by itself did not affect the biosynthesis of estradiol or progesterone (Fig. 1). However, concurrent treatment with bFGF resulted in dose-dependent inhibition of the FSH effect on estradiol production with a maximal inhibitory effect of 91 ± 1% (Fig. 1A) and on progesterone production with a maximal inhibitory effect of 41 ± 6% (Fig. 1B).

The effect of bFGF on steroidogenesis in FSH-primed granulosa cells was examined. As expected, the addition of FSH (100 ng/m*l*) to the FSH-primed cells resulted in a remarkable increase in the production of both estradiol (Fig. 2A) and progesterone (Fig. 2B). Treatment with bFGF

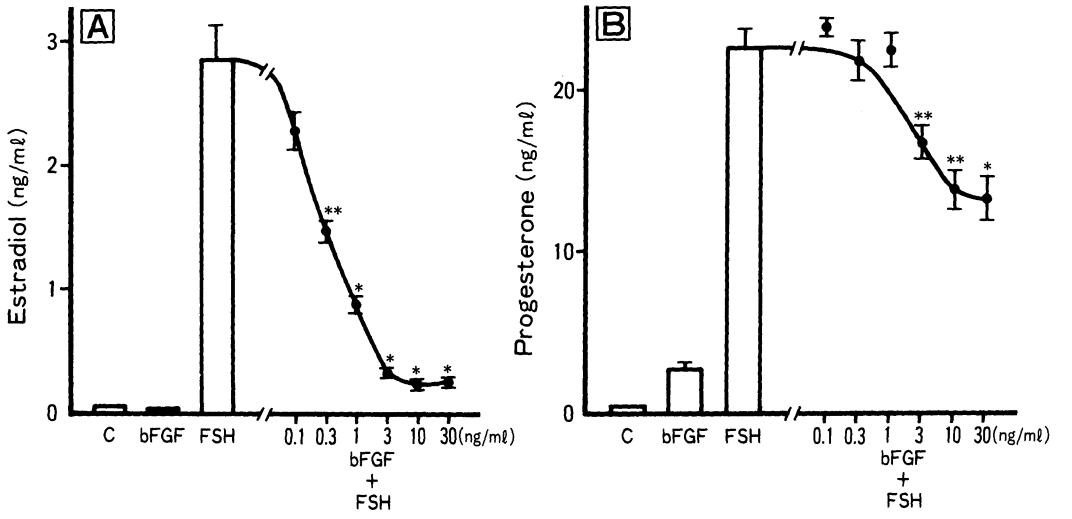

Fig. 1. Effect of bFGF on FSH-stimulated estradiol and progesterone production in rat granulosa cells. Granulosa cells (1×10^5 cells/tube) were cultured for 48 h in medium alone [control (C)] or with bFGF (30 ng/m*l*) or FSH (30 ng/m*l*) alone or FSH (30 ng/m*l*) with increasing doses of bFGF (0.1–30 ng/m*l*). At the end of the culture period, medium was collected and the concentrations of (A) estradiol and (B) progesterone were measured by specific RIA. The results represent the mean ± SEM of three separate experiments. *, $P<0.01$; **, $P<0.05$ compared with FSH-treated granulosa cells.

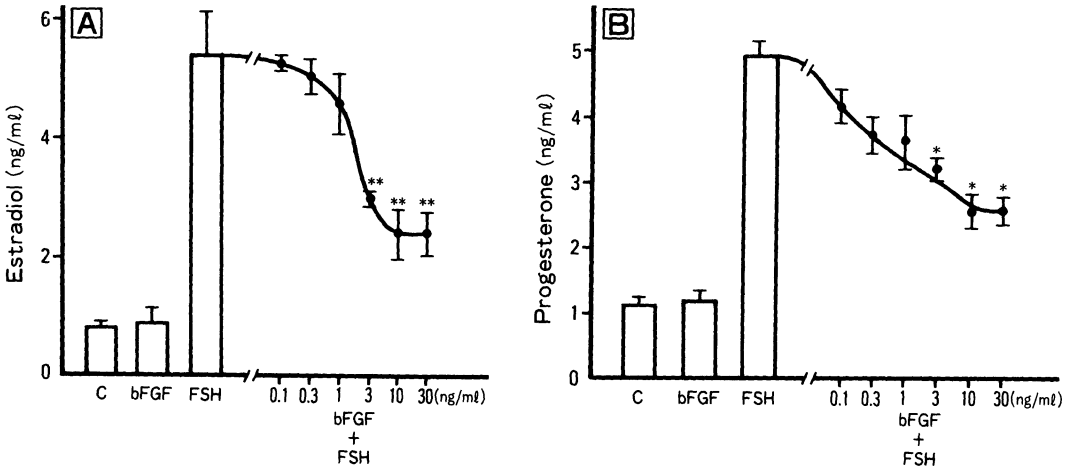

Fig. 2. Effect of bFGF on FSH-stimulated estradiol and progesterone production in FSH-primed rat granulosa cells. Granulosa cells (1×10^5 cells/tube) were initially cultured for 2 days in the presence of FSH (30 ng/m*l*), and after priming, the cells were washed twice with medium and reincubated for an additional 48 h in medium alone [control (C)] or with bFGF (30 ng/m*l*) or FSH (100 ng/m*l*) alone or FSH (100 ng/m*l*) with increasing doses of bFGF (0.1–30 ng/m*l*). At the end of the culture period, medium was collected and the concentrations of (A) estradiol and (B) progesterone were measured by specific RIA. The results represent the mean ± SEM of three separate experiments. *, $P<0.01$; **, $P<0.05$ compared with FSH-treated granulosa cells.

(30 ng/m*l*) by itself did not affect the biosynthesis of estradiol or progesterone in FSH-primed cells (Fig. 2). However, concurrent treatment with bFGF resulted in dose-dependent inhibition of the FSH effect on estradiol production with a maximal inhibitory effect of 55 ± 6% (Fig. 2A) and on progesterone production with a maximal inhibitory effect of 48 ± 5% (Fig. 2B).

We also tested the effect of bFGF on LH-stimulated steroidogenesis in FSH-primed granulosa cells. Unlike FSH, concurrent treatment with bFGF resulted in dose-dependent stimulation of

the LH effect on estradiol production with a maximal stimulatory effect of 255 ± 7% (Fig. 3A) and on progesterone production with a maximal stimulatory effect of 110 ± 35% (Fig. 3B).

Discussion

The capacity of a developing follicle to ovulate and luteinize is critically dependent upon the cytodifferentiation of granulosa cells. Increased aromatase activity and progesterone production are characteristic markers for highly differentiated granulosa cells. In the present study, we demonstrated that bFGF inhibited FSH-induced estradiol and progesterone production in both rat immature granulosa cells and FSH-primed granulosa cells. We also provided the first evidence that bFGF enhanced LH-stimulated estradiol and progesterone production in rat FSH-primed granulosa cells.

Basic FGF has been previously demonstrated to account for most of the angiogenic activity of corpus luteum extracts [8]. The presence of bFGF was demonstrated in luteal tissues, and bFGF immunoreactivity, bioactivity, and mRNA were found in bovine granulosa cells [10]. A recent study using reverse transcription-polymerase chain reaction has identified ovarian bFGF mRNA in rat ovaries [11]. In addition, specific binding sites for bFGF have been demonstrated on cultured rat granulosa cells [2, 12].

It has been reported that treatment of rat immature granulosa cells with bFGF inhibits the capacity of FSH to stimulate estrogen production [3, 13]. Our results on bFGF inhibition of FSH-stimulated estrogen biosynthesis are comparable with reports using primary culture of rat granulosa cells. On the other hand, Baird *et al.* [13] revealed that bFGF showed an apparent biphasic progesterone response to FSH in progesterone synthesis in rat granulosa cells. bFGF was found to inhibit progesterone accumulation by cultured bovine granulosa cells [14], but was reportedly without effect on progesterone production by cultured porcine granulosa cells [15]. In this study, bFGF inhibited FSH-stimulated progesterone production by both immature and FSH-primed granulosa cells. Although the reasons underlying the above discrepancies remain to be elucidated, bFGF preparations of variable quality and differing culture conditions might be involved. Our finding that recombinant bFGF is able to attenuate FSH-stimulated steroidogenesis in rat immature and FSH-primed granulosa cells, suggests that bFGF may play an inhibitory cytodifferentiative role in the ontogeny of the granulosa cell.

It is well established that FSH can induce the appearance of specific, high affinity LH receptors in isolated granulosa cells and that LH receptors

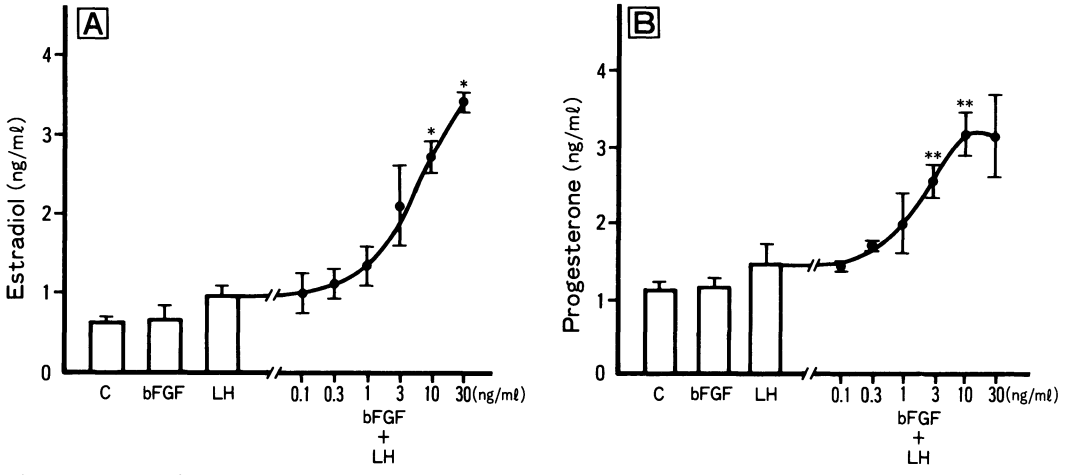

Fig. 3. Effect of bFGF on LH-stimulated estradiol and progesterone production in FSH-primed rat granulosa cells. FSH-primed granulosa cells (as described in Fig. 2) were cultured for 48 h in medium alone [control (C)] or with bFGF (30 ng/m*l*) or LH (100 ng/m*l*) alone or LH (100 ng/m*l*) with increasing doses of bFGF (0.1–30 ng/m*l*). At the end of the culture period, medium was collected and the concentrations of (A) estradiol and (B) progesterone were measured by specific RIA. The results represent the mean ± SEM of three separate experiments. *, $P<0.01$; **, $P<0.05$ compared with LH-treated granulosa cells.

are capable of mediating two important biological responses, namely, progesterone and estrogen biosynthesis [16]. This direct action of FSH in the granulosa cell can explain the increased responsiveness of the preovulatory follicle to LH during the follicular phase of the reproductive cycle. In the present study, bFGF exerted stimulatory effects on LH-induced estradiol and progesterone production in rat FSH-primed granulosa cells. Recently, Tamura *et al.* [17] reported that bFGF increased progesterone secretion in cultured rat luteal cells, and that the stimulative effect of bFGF on progesterone production gradually decreased as the corpus luteum aged.

Since the effects of FSH and LH are mediated through increased protein kinase A activity, it is difficult to explain the opposite effects of bFGF on FSH- and LH-dependent steroidogenesis in FSH-primed granulosa cells. LH activates a protein kinase C system in luteinized granulosa cells [18, 19], and ovarian follicles and corpora lutea are sites of protein kinase C activity [20]. The activation of protein kinase C in immature granulosa cells is associated with the inhibition of FSH-stimulated steroidogenesis [21, 22]. Inversely, luteinized granulosa cells respond to activation of protein kinase C with increases in cAMP production and steroid synthesis [23, 24]. Thus granulosa cells show development-related responses to activation of protein kinase C. On the other hand, it was previously shown that treatment with bFGF induced a dose-dependent increase in PGE_2 and $PGF_{2\alpha}$ production in rat luteinized granulosa cells [7, 17]. The observation that PGE_2 and $PGF_{2\alpha}$ can mimic the stimulatory effect of LH on steroid production by preovulatory follicles [25] may therefore indicate an additional mechanism through which these factors may increase steroid biosynthesis by luteinized granulosa cells.

It is uncertain whether the modulation of gonadotropin-stimulated steroidogenesis in granulosa cells by bFGF demonstrated in this study reflects the *in vivo* regulation of steroidogenesis. Since the peptide encoded by bFGF mRNA does not contain a conventional signal sequence [26], it is unclear how bFGF is released from the cell. There is evidence to suggest that bFGF polypeptides are stored in a biologically inert form bound to heparan sulfate proteoglycans in the extracellular matrix [27, 28]. Because there are no reports of bFGF levels in ovarian tissue and follicular fluid, it is not clear whether the doses of bFGF used in the present study are physiological concentrations. Recently, Guthridge *et al.* [29] reported that *in situ* hybridization signals of bFGF mRNA in cyclic rat ovary were higher in the granulosa cell layer of larger follicles when compared to smaller follicles during proestrus and estrus suggesting that bFGF mRNA levels increase throughout follicular development. They also demonstrated that *in situ* labelling of bFGF mRNA in the corpus luteum at metestrus was significantly higher than the labelling of granulosa cells in developing follicles indicating a higher level of bFGF mRNA.

In conclusion, our finding that bFGF exerts opposite effects on gonadotropin-dependent steroidogenesis suggest that bFGF may play important paracrine and/or autocrine roles in the process of follicular development, ovulation, and subsequent luteinization.

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