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The effect of activin and FSH on the differentiation of rat granulosa cells

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Abstract The differentiation of granulosa cells is regulated by follicle-stimulating hormone (FSH) and local ovarian factors. To further analyze the role of FSH and activin in this process, we have examined the effect of FSH and activin on FSH and luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor induction in granulosa cells. Granulosa cells from diethylstilbestrol (DES)-primed immature rats produce activin and maintain FSH receptor without LH/hCG receptor expression in the absence of FSH. On the other hand, FSH induced granulosa cells to differentiate into more mature granulosa cells in which higher LH/hCG receptor expression and diminished activin production were observed.

Key words: Activin; Follicle-stimulating hormone; Granulosa cell; Follicle-stimulating hormone receptor; Luteinizing hormone/human chorionic gonadotropin receptor

1. Introduction

Ovarian granulosa cells undergo a complete differentiation process during the growth and maturation of ovarian follicles. This process includes the acquisition of follicle stimulating hormone (FSH) receptors in the early stages of follicular growth, and the induction of FSH and luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors in granulosa cells is a critical step in reproductive physiology. The initial growth of granulosa cells in small follicles is independent of FSH. However, the subsequent recruitment of these follicles, which require gonadotropin support for continued growth and their selection for escape from atresia, to subsequently become dominant and ovulate, involves an interaction between gonadotropins and local regulators. Recent evidence suggests that activin may play a major role as a local regulator in ovarian follicles that both produce and respond to activins [1]. Gonadotropin receptor expression has been widely studied using a model system of primary cultures of rat granulosa cells obtained from immature female rats pretreated with estradiol [2,3]. In previous studies, we showed that activin can increase the expression of FSH receptor mRNA and proteins by accelerating transcription and by stabilizing the mRNA transcripts [4,5].

In order to study the interaction between FSH and activin as it pertains to gonadotropin receptor expression, we investigated gonadotropin receptor mRNA levels and activin secretion in cultured granulosa cells. In the present study, we have applied enzyme immunoassay (EIA) to the measurement of activin after FSH treatment in granulosa cell culture medium.

2. Materials and methods

2.1. Hormones and reagents

Activin A was kindly donated by Dr. Eto (Ajinomoto Co., Inc., Central Research Laboratories, Kawasaki, Japan). Rat FSH (I-8) was obtained from the National Hormone and Pituitary Distribution Program (Bethesda, MD). Diethylstilbestrol (DES), gentamicin sulfate, 8-bromoadenosine 3,5-cyclic monophosphate (8-Br-cAMP) and 3-isobutyl-methylxanthine were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Dulbecco's modified Eagle (DME) medium, Ham's F-12 medium, and fungizone were purchased from Gibco Laboratories (Grand Island, NY). The RNA labeling kit, nucleic acid detection kit and bisindolylmaleimide were purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Rat granulosa cell culture

Granulosa cells were obtained from immature female Wistar rats which received an injection of 2 mg diethylstilbestrol in 0.1 ml sesame oil once daily for 4 days. The ovaries were then excised, and granulosa cells were released by puncturing follicles with a 25-gauge needle. At all times, the animals were treated as humanely as possible, following NIH guidelines. Granulosa cells were washed and collected by brief centrifugation, and cell viability was determined by trypan blue exclusion. The granulosa cells were then cultured in Ham's F-12/DME (1:1, v/v) medium supplemented with 1.1 g/l $NaHCO_3$, 40 mg/l gentamycin sulfate, 1 mg/l fungizone, and 100 mg/l bovine serum albumin (BSA) on collagen coated plates in a humidified atmosphere containing 5% CO_2, 95% air at 37°C [4].

2.3. Preparation of cRNA probes

Rat FSH receptor cDNA was subcloned into the *Eco*RI site of the Bluescript KS(+) vector and linearized with *Hin*dIII [4]. Digoxigenin-labeled FSH receptor cRNA probes corresponding to bases 239–2368 were produced by in vitro transcription with T7 RNA polymerase and an RNA labeling kit (Boehringer, Mannheim). Rat LH/hCG receptor cDNA was prepared as described previously and linearized with *Bgl*II [6]. Digoxigenin-labeled LH/hCG receptor cRNA probes corresponding to bases 440–2560 were produced by in vitro transcription with T3 RNA polymerase and an RNA labeling kit (Boehringer, Mannheim). A digoxigenin labeled β-actin cRNA probe was obtained by the same method.

2.4. RNA isolation and analysis

Granulosa cells were cultured in 60-mm dishes containing 5×10^6 viable cells in 5 ml of medium, and reagents were added to the medium after 24 h of cell culture. The granulosa cells were further incubated, and the cultures were stopped at the selected time as indicated in the guanidinium acid-thiocyanate-phenol-chloroform method [7]. The final RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O. Total RNA was quantified by measuring the absorbance of samples at 260 nm. For Northern blot analysis, 15 μg total RNA from each dish were separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Biodyne, ICN). In accordance with the standard protocol for the nucleic acid detection kit (Boehringer, Mannheim), Kodak X-Omat film (Eastman Kodak, Rochester, NY) was then exposed to the membranes.

Concentrations of activin in the culture medium were determined

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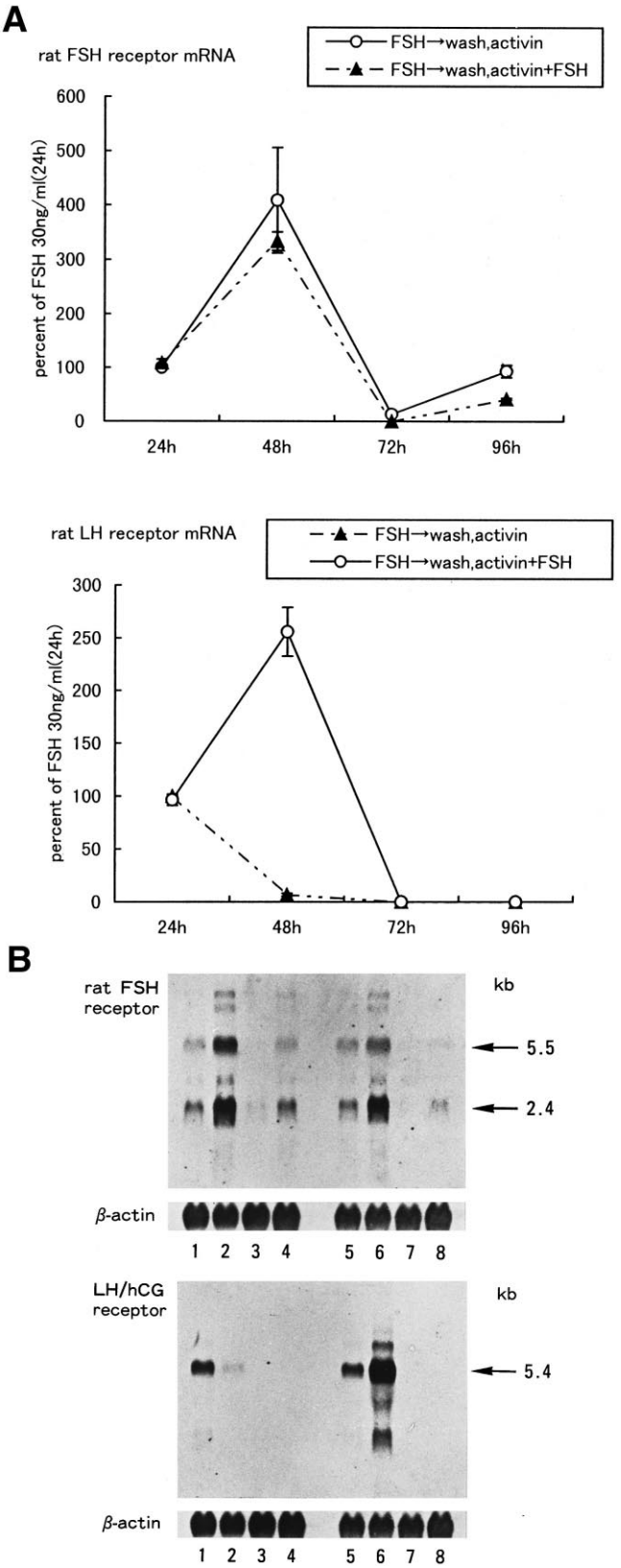
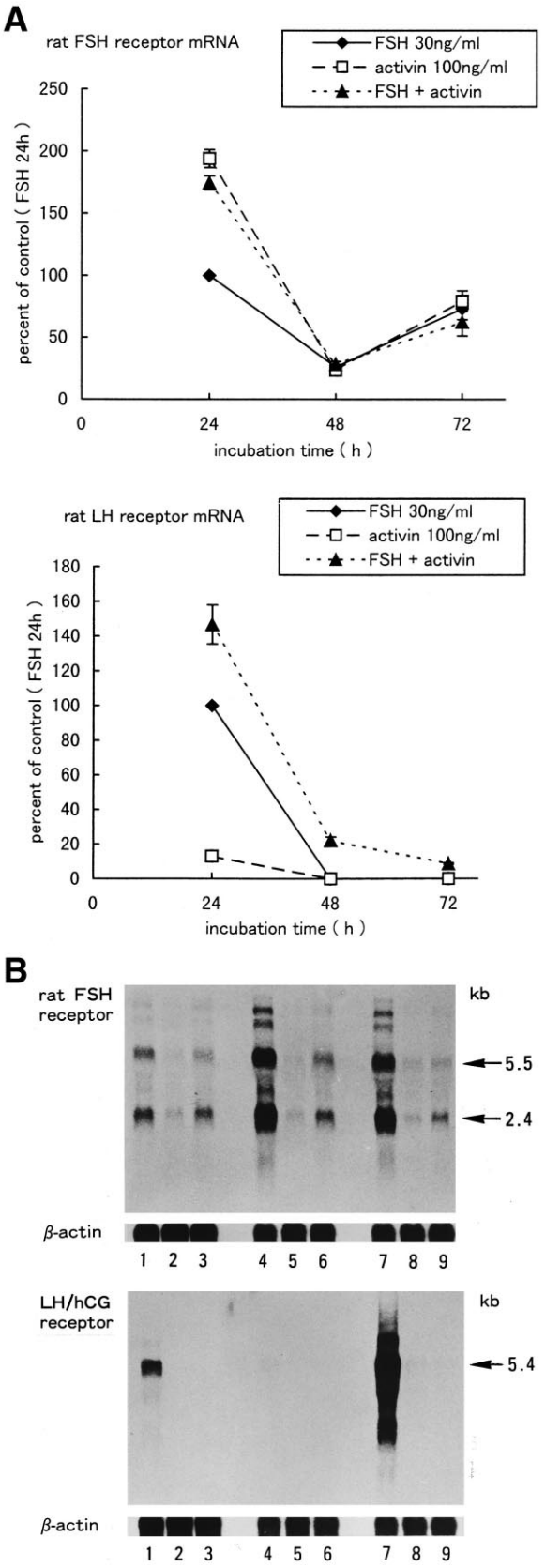


Fig. 1. Effect of FSH and activin on FSH and LH/hCG rceptor mRNA. A: Granulosa cells from DES-primed immature rats were cultured with FSH (30 ng/ml) for 24 h (lane 1), the medium was removed, and fresh medium with activin (100 ng/ml) (lanes 2,3,4) or FSH plus activin (lanes 6,7,8) was added, then the medium was again removed and the incubation was continued for the indicated times (lanes 2,6: 48 h; lanes 3,7: 72 h; lanes 4,8: 96 h). Northern blot analysis was performed as described in Section 2. B: Autoradiographs of FSH receptor mRNA (2.4 kbp) and LH/hCG receptor mRNA (5.4 kbp) were quantified by densitometric scanning. The amount of FSH receptor and LH/hCG receptor mRNA at 24 h after the addition of FSH was taken as 100%. Data were normalized for β-actin mRNA levels in each sample and expressed relative to the control value. The Northern blot is representative of three replicate experiments and the data are presented as means ± S.E.



by enzyme immunoassay (EIA) using a commercially available kit (Serotec, Oxford).

2.5. Data analysis

The relative abundance of a 2.4-kilobasepair (kbp) signal in different preparations was quantified with an LKB 2202 UnitroScan Laser Densitometer (LKB Produkter AB, Bromma, Sweden) and normalized against levels of β-actin mRNA in each sample and expressed relative to the control value (100%). For all experiments a representative Northern blot is presented of experiments performed in triplicate. The data are presented as the mean ± S.E. of measurements from triplicate cultures for one representative experiment. Differences between control and treated cells were assessed by Student's *t*-test for independent samples.

3. Results

Previous studies have shown that the continual presence of FSH is required to maintain FSH and LH/hCG receptors at elevated steady-state levels after induction [8]. To investigate the effect of activin on gonadotropin receptor levels, total mRNA from cells was prepared and analyzed using Northern blots. After treatment with FSH for 24 h, the cells were incubated with activin alone or activin plus FSH for 24 h and were washed to remove unbound hormones 24 h later. Northern blot analysis indicated two FSH receptor mRNA transcripts of approximately 5.5 and 2.4 kbp to be by far the most common in total RNA prepared from rat granulosa cells. As shown in Fig. 1, FSH receptor mRNA levels were induced by incubation with activin or activin plus FSH for 24 h and decreased following the removal of these hormones. While LH/hCG receptor mRNA levels increased with FSH treatment, subsequent addition of activin did not increase LH/hCG receptor mRNA after FSH withdrawal. However, LH/hCG receptor mRNA levels which were increased by the addition of activin plus FSH, rapidly diminished upon removal of these hormones. The loss of LH/hCG receptor mRNA that occurs when hormones are removed does not reflect a general loss of cellular mRNA since the β-actin levels remained constant after removal of the hormones.

To ascertain the specificity of the effect of these factors, the cells were incubated with FSH, activin or FSH plus activin and were washed to remove unbound factors 24 h later. FSH receptor mRNA levels fell rapidly upon removal of FSH, but had slightly recovered by 72 h. LH/hCG receptor mRNA levels fell rapidly upon removal of FSH, and were undetectable during incubation. Activin increased FSH receptor mRNA levels with a 24-h incubation, whereas there was no effect on the level of LH/hCG receptor mRNA. On the other

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Fig. 2. Induction of FSH and LH/hCG receptor mRNA by FSH, activin and FSH plus activin. Granulosa cells from DES-primed immature rats were cultured with FSH (30 ng/ml), activin (100 ng/ml) and FSH plus activin for 24 h (lanes 1,4,7), the medium was removed, and the incubation was continued for the indicated times (lanes 2,5,8: 48 h; lanes 3,6,9: 72 h). Northern blot analysis was performed as described in Section 2. B: Autoradiographs of FSH receptor mRNA (2.4 kbp) and LH/hCG receptor mRNA (5.4 kbp) were quantified by densitometric scanning. The amount of FSH receptor and LH/hCG receptor mRNA at 24 h after FSH addition was taken as 100%. Data were normalized for β-actin mRNA levels in each sample and expressed relative to the control value. The Northern blot is representative of three replicate experiments and the data are presented as means ± S.E.

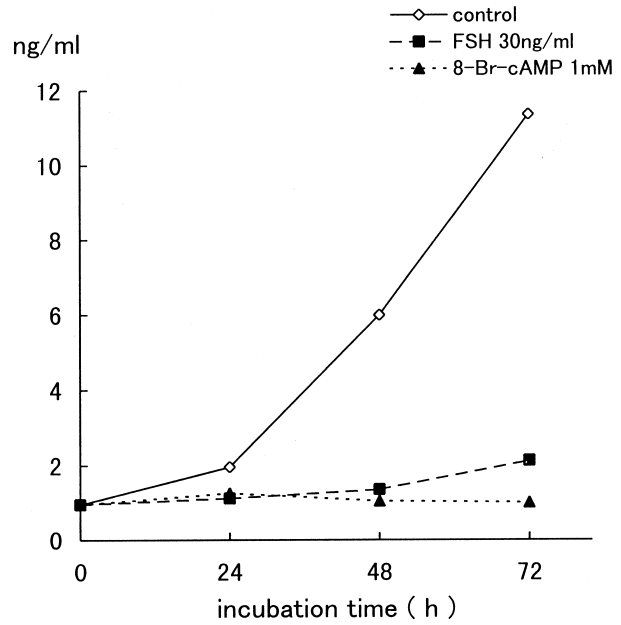

Fig. 3. Concentration of activin in the medium. Granulosa cells from DES-primed immature rats were cultured for 24 h and then treated with and without FSH (30 ng/ml). The media were removed at the indicated times and activin levels were measured by EIA. The results are shown as the mean ± S.E.

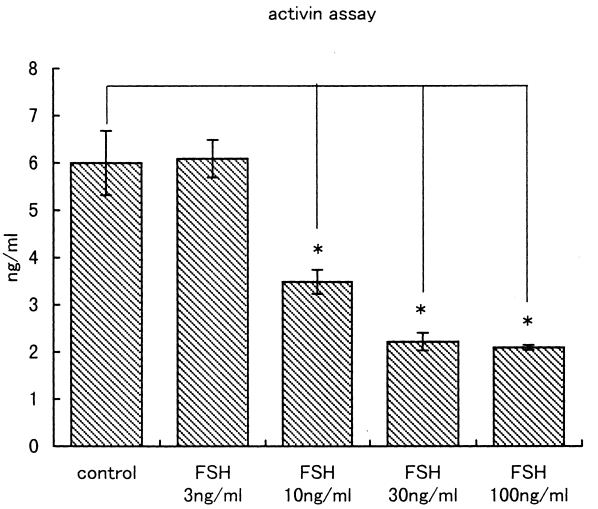

Fig. 4. Dose-related inhibition of activin production in culture medium by FSH. Granulosa cells from DES-primed immature rats were cultured for 24 h and then treated with FSH at the indicated concentrations. The activin levels in the medium at 48 h incubation were measured using EIA. The results are shown as the mean ± S.E. *Different from the value of control at $P < 0.01$.

hand, activin plus FSH increased FSH receptor mRNA levels to those induced by FSH alone, while strongly inducing LH/hCG receptor mRNA levels (Fig. 2). Although activin has a strong effect on the expression of gonadotropin receptor mRNA, its effect is dependent on the duration of granulosa cell incubation. The effect of each treatment diminished upon removal of unbound hormones. These data show that activin induces FSH receptor expression by itself and increases LH/hCG receptor mRNA levels in the presence of FSH.

Since activin stimulated FSH receptor induction in rat granulosa cells, we examined the effect of FSH on the production of activin in the culture medium. As shown in Fig. 3, there appeared to be an increase in the basal level of activin as the cells were cultured for increasing lengths of time in the absence of stimuli. After a 24-h incubation with FSH (30 ng/ml), there was no detectable increase in activin levels above that observed at time 0 in culture. During incubation, the addition of FSH decreased the activin level in the medium. To determine whether the effect of FSH is mediated by the cAMP/A kinase pathway, cells were incubated with 8-Br-cAMP (1 mM). The addition of 8-Br-cAMP decreased the activin level in the medium showing a time course similar to that seen with FSH. In addition, the time course showed that removal of FSH or cAMP resulted in recovery of activin levels in the medium.

As shown in Fig. 4, activin levels in the medium decreased dose-dependently when incubated for 48 h with FSH, and 10 ng/ml of FSH significantly decreased activin levels as compared with the control.

4. Discussion

The results of previous nuclear run-on or Northern blot experiments demonstrated that activin stimulates the rate of transcription of the FSH receptor gene in granulosa cells of DES-primed immature rats [4,5]. Fig. 1 shows that the addition of activin increased the FSH receptor level. This was true even if the medium, which includes FSH, was changed. Thus, these data demonstrate that the signal transduction of activin differed from that of FSH in regulating the FSH receptor. The removal of medium including activin reduces expression of the FSH receptor, and at 24 h after medium removal the FSH receptor mRNA levels are markedly lower than the control level. Therefore, the granulosa cells are not able to undergo a 'committed step' after the activin-mediated induction of FSH receptor mRNA.

On the other hand, FSH induced LH/hCG receptor mRNA with a 24-h incubation, while activin had no effect on the LH/hCG receptor mRNA level after FSH withdrawal. These data show that the coexistence of FSH and activin enhances LH/hCG receptor expression and that activin treatment subsequent to the addition of FSH has no effect on LH/hCG receptor expression. We speculate that treatment with FSH plus activin induces FSH receptor expression via activin, and the following enhancement of FSH effects through these FSH receptors, leading to the induction of LH/hCG receptors. In general, high expression of the FSH receptor is accompanied by LH/hCG receptor expression in granulosa cells. However, according to the activin experiment results, activin induces high FSH receptor mRNA expression alone, i.e. without LH/hCG receptor expression. Since LH/hCG receptor expression is related to the differentiation of granulosa cells, activin is an important factor in maintaining granulosa cells in an undifferentiated immature state. On the other hand, the existence of FSH causes differentiation of granulosa cells, regardless of the presence or absence of activin. FSH plus activin cause very high LH/hCG receptor expression with a slight increase in FSH receptor expression. These results show that a discrepancy in the expression of FSH and LH/hCG receptors can be observed by the treatment of these factors, and further study is required to elucidate these mechanisms.

The levels of secreted inhibin [9,10] and of inhibin mRNA [11–13] were positively regulated by FSH in primary cultures

of rat granulosa cells, consistent with in vivo data indicating that FSH is an important regulator of inhibin gene expression and protein secretion [14,15]. In addition, FSH directly increased the transcription rates of the inhibin α and βA genes in immature rat granulosa cells [16]. The stimulatory effects of gonadotropins on inhibin secretion and subunit mRNA levels were mimicked by cAMP and cAMP-generating reagents. These results suggest that stimulation of inhibin gene expression and protein biosynthesis is through the cAMP/kinase A pathway. Taken together with present data, the increase of inhibin and decrease of activin production induced by FSH is through the cAMP/kinase A pathway. Therefore, the reduction of activin levels induced by FSH in this culture system may be due to the consumption of βA subunit for production of inhibin.

Recent evidence suggests that activin may play a major role as a local regulator in ovarian follicles that both produce and respond to activin [1]. The expression of activin subunit mRNA in granulosa cells is limited in growing follicles, but not in atretic follicles [17]. The development of large follicles is stimulated by and depends on FSH. However, the initial growth of granulosa cells in small follicles is independent of FSH and is observed in hypophysectomized animals. In previous experiments it was found that activin supported cell survival and cell proliferation of the rat granulosa cell line and maintained the functional FSH receptor in these cells throughout long term culture in the absence of FSH [18]. In this study, we found that FSH decreases activin levels in culture medium and that this decrease in activin levels may be of relevance to the maturation of granulosa cells together with initiating LH/hCG receptor expression.

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