FEBS Letters 402 (1997) 198–202 FEBS 18134

Down-regulation of LH/hCG receptor in rat cultured granulosa cells

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Received 18 November 1996

Abstract Initial experiments established the experimental conditions necessary for the induction of LH receptor mRNA down-regulation in granulosa cells isolated from diethylstilbestrol (DES)-primed immature rats. LH/hCG receptor mRNA was first induced in granulosa cells by incubating them for 24 h with FSH. Exposure of granulosa cells to a second dose of gonadotropin caused a decrease in LH/hCG receptor mRNA and binding levels after 4 h. This effect was transient, by 12 h the mRNA levels had returned to control levels, and by 24 h the mRNA levels were higher than the control. To evaluate the ability of cAMP to down-regulate the receptor, cells were exposed to 8-Br-cAMP. The pattern of sustained decrease in LH/hCG receptor mRNA levels seen with 8-Br-cAMP was similar to that with gonadotropins. The presence of activin with FSH or hCG is more effective than FSH or hCG alone in inducing LH/hCG receptor down-regulation in rat granulosa cells. The LH/hCG receptor mRNA levels decreased in a dose-dependent manner in the presence of increasing concentrations (30–100 ng/ml) of activin with FSH. These observations indicate that an increase in cAMP causes down-regulation of LH/hCG receptor mRNA and contributes to the effect of FSH and hCG, whereas basal cAMP activity is required for LH/hCG receptor mRNA expression.

Key words: Luteinizing hormone receptor; Follicle stimulating hormone; Down-regulation; Cyclic AMP

1. Introduction

The pseudopregnant rat model has been used to investigate correlations between LH/hCG receptor mRNA levels and the expression of receptor sites during various stages of ovarian differentiation and receptor down-regulation. The administration of an ovulatory dose of hCG causes a temporary loss of LH/hCG receptors in ovulated follicles. A secondary increase in LH/hCG receptors after induction of ovulation by hCG occurs during follicular luteinization and formation of corpora lutea [1]. The concentration-dependent and developmental stage-specific actions of gonadotropins indicate the complexity of the regulatory mechanisms operating in gonadal target cells.

The mechanisms involved in the down-regulation of gonadotropin receptor are difficult to study in vivo. A model system to study the effect of gonadotropin on granulosa cells obtained from immature female rats that have been pretreated with estradiol [2,3] appears to provide a new approach to this problem. Since the down-regulation of LH/hCG receptor has not been observed in this defined system, the present studies were undertaken to establish the conditions for induction of LH/hCG receptor down-regulation in rat granulosa cells. In order to observe the down-regulation induced by gonadotropins, we preincubated the granulosa cells with FSH for 24 h before further addition of gonadotropins. Our results have also demonstrated the coordinate effects of gonadotropin and activin on up- and down-regulation of LH/hCG receptor in cultured rat granulosa cells.

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 (NIH, FSH, I-8) and hCG (CR-127) were provided by the National Pituitary Agency. Diethylstilbestrol (DES), gentamicin sulfate, 8-bromo-adenosine 3,5-cyclic monophosphate (8-Br-cAMP) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), Ham's F-12 medium, and fungizone were purchased from Gibco Laboratories (Grand Island, NY). The RNA labeling kit and nucleic acid detection kit were purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Rat granulosa cell culture

Immature female Wistar rats (21 days old) were obtained from Imai Experimental Animal Farm (Saitama Prefecture, Japan). Since estrogen treatment in vivo results in the formation of multiple preantral follicles and provides a large number of relatively homogeneous cells at the same stage of development, rats were given daily subcutaneous injections of 2 mg of DES in 0.1 ml sesame oil for 4 days. The rats were killed at 26 days and their ovaries were aseptically excised. All procedures followed NIH guidelines.

Granulosa cells were obtained by puncturing ovaries from DES-treated immature rats. The granulosa cells were then cultured in Ham's F-12/DMEM (1:1 v/v) medium supplemented with 1.1 g/l $NaHCO_3$, 40 mg/l gentamicin sulfate, 1 mg/l fungizone, and 100 mg/l BSA in a humidified atmosphere containing 5% CO_2, 95% air at 37°C.

2.3. Receptor binding assay

These experiments were performed with cells plated in Immulon 2 Removawell (Dynatech Laboratories, Inc., Alexandria, VA). Each well contained 1×10^5 cells in 100 μl medium. Highly purified hCG was iodinated according to the chloramine-T method. To measure the LH/hCG receptor content, the granulosa cell monolayers were incubated for 2 h at 37°C with [^{125}I]hCG (0.4 ng, 100 000 cpm/ng) in the absence or presence of 50 IU crude hCG. At the end of the incubation, the cells were set on ice and washed three times with 200 μl assay medium. Each well was then torn off the Removawell strip and the amount of radioactivity remaining in the well (cell-bound hormone) was measured with a γ-spectrometer.

2.4. Preparation of cRNA probe

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

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PII S0014-5793(96)01528-1

2.5. 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 guanidium acid-thiocyanate-phenol-chloroform method [5]. The final RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O and total RNA was quantified by measuring the absorbance of samples at 260 nm. For Northern blot analysis, 15 mg of total RNA from each dish was separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Biodyne, ICN). Northern blots were prehybridized for 6 h at 68°C and then hybridized overnight at 68°C with digoxigenin-labeled cRNA probes. 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. Luminescence detection was quantified with an LKB 2202 Unitro Scan Laser Densitometer (LKB Produkter AB, Bromma, Sweden), normalized against a corresponding relative amount of β-actin mRNA in each sample, and expressed as relative densitometric units.

The data are presented as means ± S.E.M. of measurements from triplicate separate experiments. Differences between control and treated cells were assessed by Student's *t*-test for independent samples.

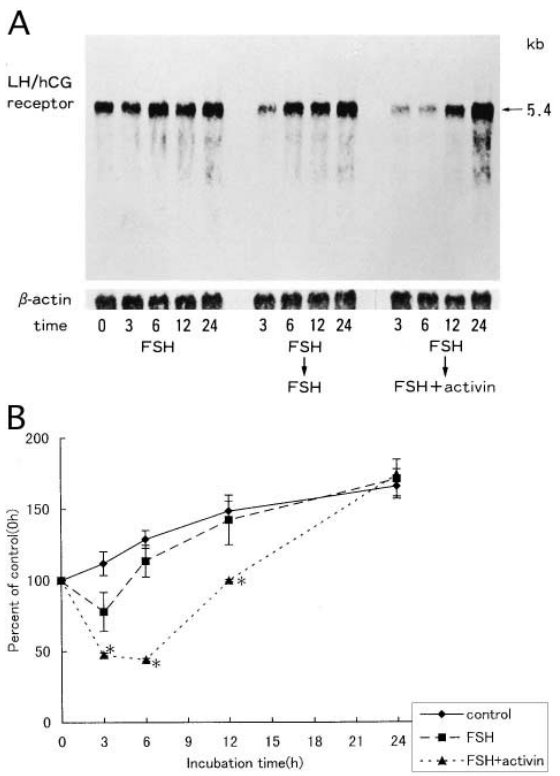

Fig. 1. Time course of change in LH/hCG receptor mRNA levels. A: Cells were incubated for 24 h in the presence of FSH (30 ng/ml), and further incubation was performed for 24 h in the presence or absence (control) of freshly added 30 ng/ml FSH or 30 ng/ml FSH and 100 ng/ml activin. Total RNA was extracted from granulosa cells at selected times after 24 h incubation of cells in the presence of FSH. Northern blots were performed to analyze of LH/hCG receptor mRNA with LH/hCG receptor cRNA probe. B: Autoradiographs of LH/hCG receptor mRNA (5.4 kb) were quantified by densitometric scanning. 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 the mean ± S.E.M. *Different from the control value at $P<0.01$.

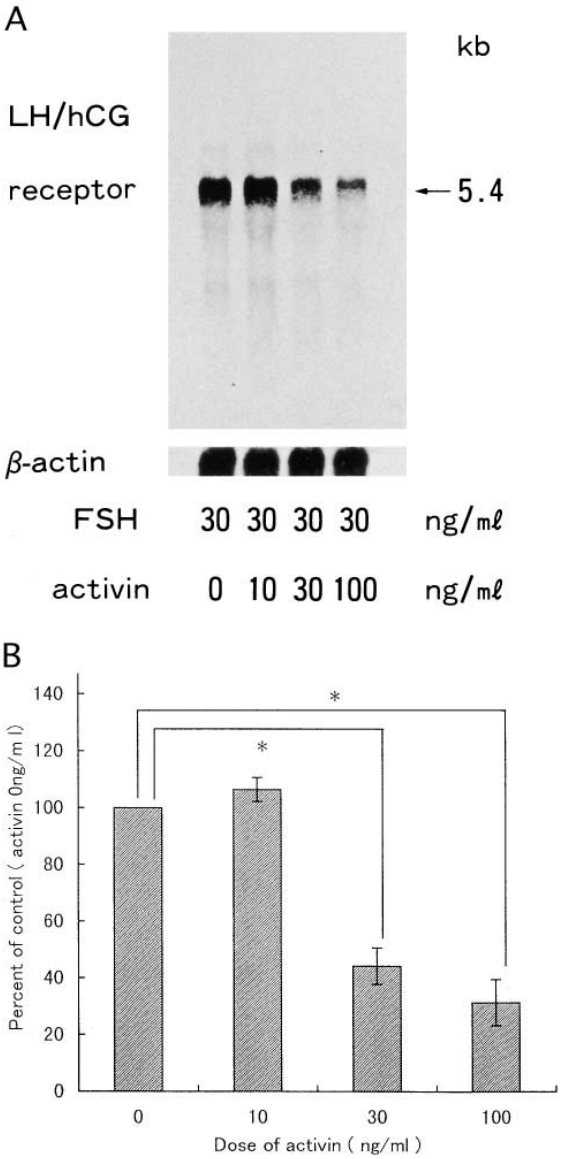

Fig. 2. Cooperative effect of activin of FSH-induced transient down-regulation of LH/hCG receptor mRNA levels. A: Total RNA was prepared from cells incubated for 6 h, after 24 h pre-incubation in the presence of 30 ng/ml FSH or 30 ng/ml FSH with increasing concentrations of activin, and was analyzed by Northern blot with an LH/hCG receptor cRNA probe. The Northern blot is representative of three replicate experiments. B: Autoradiographs of LH/hCG receptor mRNA (5.4 kb) were quantified by densitometric scanning. 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 the mean ± S.E.M. measurements. *Different from the control value (activin 0 ng/ml) at $P<0.01$.

3. Results

Initial experiments were designed to establish the experimental conditions necessary for the induction of LH/hCG receptor mRNA down-regulation in granulosa cells isolated from DES-primed immature rats. LH/hCG receptor mRNA expression was induced by incubating cells for 24 h with FSH. As shown in Fig. 1A, the incubation of these cells with FSH for 24 h resulted in the induction of LH/hCG receptor mRNA to levels comparable to previous reports [6–9]. Previous stud-

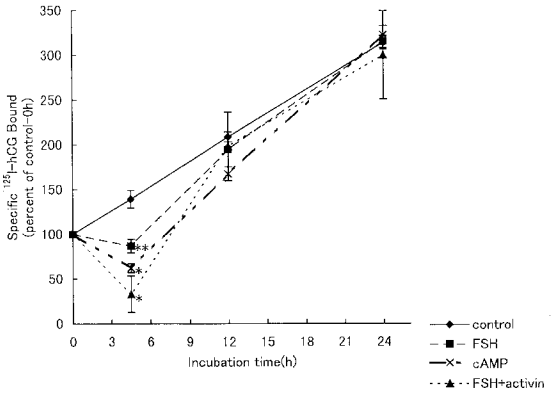

Fig. 3. Granulosa cells from DES-primed immature rats were cultured with 30 ng/ml FSH and at 24 h (t = 0) cells received either another 30 ng/ml FSH or 0.5 mM 8-Br-cAMP or nothing (control). After various incubation times, the levels of LH receptor were determined by [^{125}I]hCG binding assays. One experiment representative of five independent experiments is shown. Values in panel are the mean ± S.E.M. of triplicate determinations. *Different from the control value at $P < 0.01$. **Different from the control value at $P < 0.05$.

ies have described that the continual presence of FSH is required to maintain the LH/hCG receptor at elevated steady-state levels after induction [10]. Therefore the cells were incubated with FSH for the first 24 h, and were then incubated for another 24 h in the presence or absence of freshly added FSH. As shown in Fig. 1A, without the second addition of 30 ng/ml of FSH, a slight increase in FSH-stimulated LH receptor mRNA levels was observed. In contrast, LH/hCG receptor mRNA decreased after the addition of another dose of FSH at 0 h, and there was a rapid and transient decrease in LH/hCG receptor mRNA.

We previously reported that incubation of granulosa cells with optimal concentrations of activin and FSH resulted in induction of LH/hCG receptor mRNA and protein. The addition of activin with FSH might therefore be expected to prevent FSH-induced LH/hCG receptor down-regulation. However, as shown in Fig. 1A, the presence of activin was more effective than FSH alone in inducing LH/hCG receptor mRNA down-regulation in rat granulosa cells. There was a rapid decrease in levels of LH/hCG receptor mRNA expression, declining to 50% of control values between 3 h and 6 h incubation. The dose-dependent effects of activin on LH/hCG receptor mRNA levels were examined in cells incubated for 6 h with FSH and activin. The LH/hCG receptor mRNA levels decreased in a dose-dependent manner compared to the control in the presence of increasing concentrations (30–100 ng/ml) of activin with FSH (Fig. 2A).

Decreased binding activity was observed 4 h after addition of the second stimulus compared to the control, after which the binding activity increased rapidly to control levels by 24 h. By 24 h, the binding activity showed parallel changes to LH/hCG receptor mRNA levels (Fig. 3). Exposure of granulosa cells to hCG caused a decrease in LH/hCG receptor mRNA levels (Fig. 4A). As with FSH, this effect was transient, such that by 12 h mRNA levels had returned to control levels, and by 24 h mRNA levels were higher than the control. Exposure of granulosa cells to hCG with activin caused a sustained decrease in LH/hCG receptor mRNA levels, and a rapid increase of mRNA levels after the down-regulation. Since we have previously observed FSH- and LH-stimulated cAMP production in these granulosa cells, cells were exposed to 8-Br-cAMP to determine whether cAMP could down-regulate LH/hCG receptor mRNA levels. The pattern of sustained decrease in LH/hCG receptor mRNA levels with 8-Br-cAMP was similar to that induced by hCG (Fig. 4A). These observations indicate that an increase in cAMP causes a down-regulation of LH/hCG receptor mRNA and accounts for the effects of FSH and hCG, whereas basal cAMP activity is required for LH/hCG receptor mRNA expression.

4. Discussion

In previous experiments, hCG induced down-regulation of LH/hCG receptor mRNA levels was followed by down-regulation of membrane receptor levels in the pseudopregnant rat ovary [4,11,12], indicating that the loss of hCG binding seen during hCG-induced down-regulation was, at least in part, due to the loss of receptor mRNA. Expression of the LH/hCG receptor in the rat ovary is under complex hormonal control. The present studies were undertaken to study the mechanisms underlying the down-regulation of the LH/hCG receptor in the granulosa cells. As a first step toward determining the transcriptional and translation controls governing these changes in receptor expression, we have established an experimental model of LH/hCG-induced LH/hCG receptor down-regulation in a cell culture system.

Consistent with previous studies [9,10], our data show that FSH is able to induce LH/hCG receptor and LH/hCG receptor mRNA expression in granulosa cells from DES-primed immature rats from almost undetectable basal levels (Fig. 1).

In this defined system, the ability of FSH to stimulate the induction of LH/hCG receptor is mediated, at lease in part, by cAMP since exogenously added cAMP or other agents that increase intracellular levels of cAMP mimic the actions of FSH [6,10,13,14].

There is a lag time of at least 24 h after FSH addition before increases in either LH/hCG receptor or LH/hCG receptor mRNA levels [15]. Thus a second addition of FSH after the increase of LH/hCG receptor was performed to see the down-regulation of LH/hCG receptor in the cultured granulosa cells. On the other hand, it has been shown in MA-10 Leydig tumor cells that 8-Br-cAMP causes a decrease in the rate of transcription of the LH/hCG receptor gene, resulting in decreased levels of LH/hCG receptor mRNA [16]. In addition, the rapid loss of LH/hCG receptor mRNA in pre-ovulatory follicles that occurs in response to an ovulatory dose of LH/hCG in vivo [12] or in vitro demonstrated that high levels of LH/hCG have inhibitory effects on the transcription of the LH/hCG receptor gene and/or on mRNA stability. FSH, hCG or 8-Br-cAMP added to this granulosa cell culture transiently decreased LH/hCG receptor mRNA levels which was followed by a subsequent increase in message levels. As cAMP is the established second messenger that mediates FSH-induced granulosa cell differentiation, it was of interest to evaluate the effect of cAMP on LH/hCG receptor mRNA. The time courses and magnitude of the effect of gonadotropins and 8-Br-cAMP on LH/hCG receptor mRNA were found to be comparable.

These data support the idea that the FSH- or hCG-induced loss of LH/hCG receptor mRNA in granulosa cells is mediated by cAMP. These observations suggest that a labile desta-

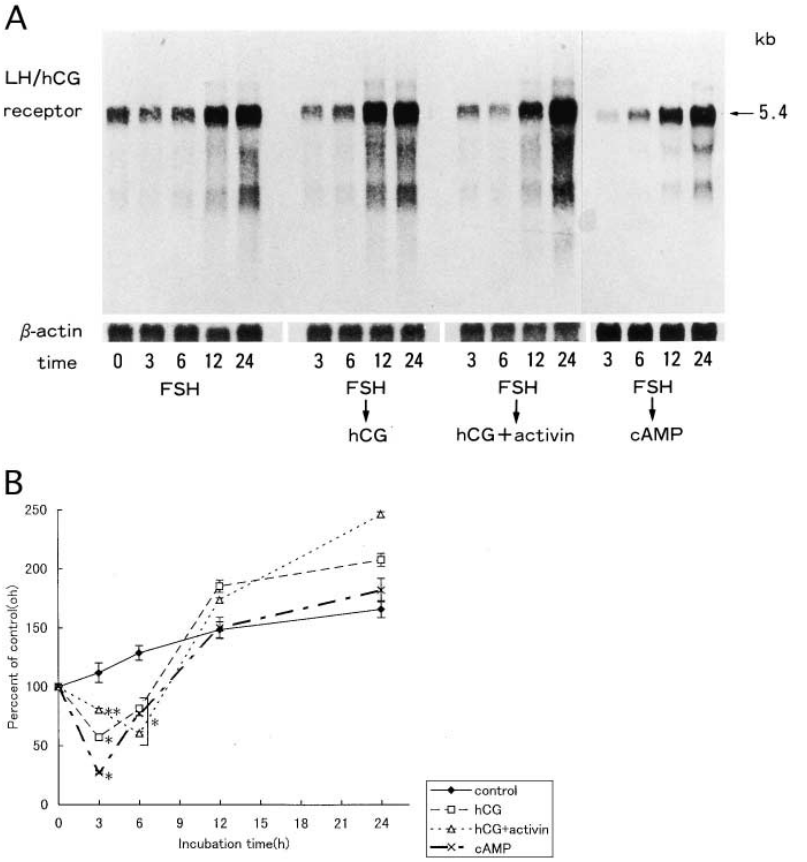

Fig. 4. Time course of change in LH/hCG receptor mRNA levels. A: Granulosa cells from DES-primed immature rats were cultured continuously with 30 ng/ml FSH only (control) or another 30 ng/ml hCG or 30 ng/ml hCG with 100 ng/ml activin or 0.5 mM 8-Br-cAMP added at 24 h (t = 0). After various incubation times, total RNA was extracted from granulosa cells. Northern blots were performed to analyze the levels of LH/hCG receptor mRNA with LH/hCG receptor cRNA probe. B: Autoradiographs of LH/hCG receptor mRNA (5.4 kb) were quantified by densitometric scanning. 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 the mean ± S.E.M. *Different from the control value at $P < 0.01$ **Different from the control value at $P < 0.05$.

bilizing factor which is induced by cAMP may degrade the LH/hCG receptor mRNA. The existence of such a destabilizing factor might explain the rapid decrease and increase after the transient decrease in mRNA levels. Since there was a long lag time before FSH- or 8-Br-cAMP-stimulated increases in LH/hCG receptor gene transcription, the LH/hCG receptor gene appears to be an unusually slow response gene probably requiring the synthesis of a new transactivation factor for expression. According to the time course of changes in LH/hCG receptor mRNA levels, FSH, LH and cAMP may act to increase LH/hCG receptor mRNA levels and simultaneously might increase levels of the destabilizing factor.

The increase in LH/hCG receptor mRNA after 6 h incubation could be because cAMP sustains the increase in LH/hCG receptor gene transcription for longer than the increase in destabilizing factors. Although the changes in LH/hCG receptor gene transcription elicited by 8-Br-cAMP suggest that FSH and LH/hCG mediate this effect through the cAMP-dependent protein kinase pathway, we cannot rule out the possible involvement of other signal transduction pathways. The LH/hCG-induced loss of LH/hCG receptor mRNA occurs relatively quickly and hormone-induced changes in the numbers of LH/hCG receptors in the rat ovary were associated with changes in the amounts of relative abundance of LH/hCG receptor mRNA transcripts.

Activin increased the responsiveness of granulosa cells to both gonadotropins [17,18]. These in vitro observations are consistent with activin acting as an autocrine regulator to promote folliculogenesis during the preantral or early antral stage because these data were all obtained from the immature estrogen-treated rat model in which the granulosa cells are not fully differentiated. On the other hand, the action of activin on differentiated granulosa cells is consistent with a role in luteinization inhibition [19]. In particular, partially differentiated granulosa cells were obtained from DES- and FSH-treated immature rats, in which activin stimulated basal progesterone production but inhibited FSH-stimulated progesterone production in vitro [18]. When similar experiments were performed on granulosa cells obtained from DES-treated rats that received both FSH and hCG in vivo and were therefore fully differentiated, both basal and FSH-stimulated progesterone production were inhibited by activin [20]. Moreover, recombinant human activin A causes a time- and dose-dependent inhibitory effect on LH-induced production of progesterone, by partially differentiated bovine granulosa cells [21]. These results are reversible, indicating that the inhibitory actions of activin are not due to a reduction in cell number. Since in this experiment activin sustained the decrease of LH/hCG receptor induced by gonadotropins, it was suggested that activin has a negative effect on LH action in cultured

granulosa cells through the down-regulation of the LH/hCG receptor. The data from the rat and bovine models support the hypothesis that activin regulates granulosa cells in an autocrine fashion and that the action of activin is related to the stage of follicular maturity.

Acknowledgements: This work was supported by a grant from the Ministry of Education, Science and Culture of Japan (05671353), Tokyo, Japan.

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