Regulation of Gonadotropin Releasing Hormone Receptor mRNA Expression in Cultured Rat Granulosa Cells

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The homologous regulation of pituitary Gonadotropin Releasing Hormone Receptor (GnRH-R) mRNA expression by GnRH has been well demonstrated. However, the regulation of the ovarian GnRH-R is poorly understood. The present study was performed to demonstrate the presence of GnRH transcripts in addition to GnRH-R mRNA and the regulation of GnRH-R mRNA expression in the granulosa cells isolated from small antral follicles. The GnRH and GnRH-R mRNA levels were determined by a competitive reverse transcription-polymerase chain reaction (RT-PCR). The granulosa cells were obtained from immature rats implanted with diethylstilbestrol for 3 days. When GnRH transcript expression was examined in isolated granulosa cells by RT-PCR, the PCR products showed two bands. The larger band contained intronic sequences and the smaller band was a fully processed GnRH gene transcript identical to hypothalamic GnRH. This suggests that authentic GnRH gene transcripts are expressed in ovarian granulosa cells and may act on the granulosa cells in a paracrine or autocrine manner. Since GnRH action in the granulosa cells is mediated by specific GnRH-R, it is of interest to examine whether GnRH-R is synthesized in the granulosa cells. When the granulosa cells were cultured in media only, GnRH-R mRNA levels increased abruptly within 3 h and gradually decreased thereafter during the 24 h culture period. However, GnRH itself did not alter the GnRH-R mRNA expression levels in cultured granulosa cells. Interestingly, treatment with FSH decreased the GnRH-R mRNA levels in a dose-dependent manner. A time-course analysis revealed that the GnRH-R mRNA levels were significantly lower up to 9 h after FSH treatment, and returned to the basal level between 12 h~24 h. Activation of adenylate cyclase with forskolin also decreased the GnRH-R mRNA levels. It is therefore concluded that in the granulosa cells of the small antral follicles GnRH-R mRNA expression was not homologously regulated by GnRH, while FSH may negatively regulate GnRH-R mRNA expression in the granulosa cells possibly through a cAMP-protein kinase A pathway.

Key Words: GnRH receptor mRNA, granulosa cell, competitive RT-PCR

INTRODUCTION

The gonadotropin releasing hormone (GnRH), a hypothalamic decapeptide, plays a crucial role in regulating mammalian reproduction. GnRH released from the hypothalamic neurons stimulates the pituitary to synthesize and secrete gonadotropins, LH and FSH, which regulate the steroidogenesis and gametogenesis within the gonads.

However, there is evidence that GnRH is produced in placenta,[1-3] breast,[4,5] gonads,[6-8] and immune system.[9] The GnRH receptor (GnRH-R) gene is also expressed in a variety of organs besides the anterior pituitary gland, suggesting an extrapituitary action of GnRH. A number of biological responses have been observed upon activation of GnRH-R in the ovaries. GnRH action in the granulosa cells appears to vary with different stages of follicular maturation.[10-12] GnRH induces oocyte maturation in preovulatory follicles[10] and follicular rupture.[11] On the contrary, the effect of GnRH on the smaller follicles seems to be inhibitory in nature, as GnRH analogs decrease the steroidogenesis and the number of gonadotropin receptors.[12] GnRH may also play a role in inducing follicular atresia in the rat ovary, since GnRH agonists induce apoptotic cell death in the granulosa cells and stimulate the production of the insulin-like growth factor-binding protein 4 (IGF-BP4), a marker for follicular

atresia.[13,14]

GnRH exerts its action through binding to the membrane receptors in specific target cells. It has been demonstrated that GnRH itself homologously regulates the number of GnRH-R in the pituitary gland, the primary target organ of hypothalamic GnRH. Low concentration or pulsatile administration of GnRH up-regulate GnRH-R mRNA levels as well as the number of receptors.[15] On the contrary, high concentration or continuous treatment with GnRH results in the down-regulation of the GnRH receptor in the pituitary gland,[16,17] although we previously reported that even continuous GnRH treatment for a relatively short period up-regulates GnRH-R mRNA.[18]

However, the mechanism by which GnRH-R gene is regulated in the ovaries is poorly understood. Only few *in vitro* studies on preovulatory granulosa cells or granulosa-luteal cells are available.[8,19] In those studies, GnRH-R gene expression has been shown to be homologously up-regulated at a confined GnRH concentration in the ovarian cells. Besides, Olofsson et al.[19] reported that the GnRH-R gene was also heterologously regulated by LH, but not FSH. Therefore, the present study was performed to determine whether GnRH-R mRNA expression in the ovaries is homologously regulated by GnRH itself as in the pituitary gland or what other factors are involved in regulating GnRH-R mRNA expression in cultured granulosa cells isolated from the small antral follicles.

MATERIALS AND METHODS

Preparation and culture of preantral granulosa cells

Immature female Sprague-Dawley rats (21 days old), obtained from Yuhan Research Center (Anyang, Kyungki-Do, Korea), were housed under controlled environmental conditions and had free access to standard pellets and filtered water.

Silastic capsule (10 mm) containing diethylstilbestrol (DES, 10 mg/rat, SC) was implanted at the age of 24 days. Seventy-two hours later, animals were killed by decapitation and the ovaries were removed, free of fat and connective tissues. The granulosa cells that were obtained by needle puncture from the ovaries with the aid of 26-gauge syringe needle were cultured in a 12-well culture plate (Costar, Corning, NY, USA).[20] Each well contained 1×10^6 viable cells (determined by trypan blue exclusion) in a 1 ml serum-free McCoy's 5A culture medium (10 mM HEPES, 2 mM L-glutamine and antibiotics of 100 units penicillin and 100 μg streptomycin/ml) with and without hormones. Each hormone was added at the beginning of culturing and the cells were maintained at 37°C in an atmosphere of 95% air-5% CO_2 for the indicated time.

The cultured cells were then washed with Dulbecco's phosphated buffered saline (D-PBS) twice and then scraped from the wells with the aid of a rubber policeman to determine GnRH-R mRNA levels.

All reagents, if not otherwise stated, were purchased from Sigma (St Louis, MO, USA).

Total RNA extraction

The total RNA from the cultured granulosa cells was extracted using the acid guanidinium thiocyanate-phenol-chloroform method.[21] After the treatment with a 500 μl denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauroyl sarcosine, and 0.1 M 2-mercaptoethanol), the granulosa cells lysate in each well was transferred to a microcentrifuge tube. Fifty microliters of 2 M sodium acetate (pH 4), 500 μl of water-saturated phenol, and 100 μl of chloroform/isoamyl alcohol mixture (49:1) were added and mixed. After cooling on ice for 15 min, samples were centrifuged (10,000 $\times$ g) at 4°C for 20 min and precipitated with ethanol. After washing with 75% ethanol, the RNA pellet was dried under vacuum and dissolved in 20 ml of sterilized diethylpyrocarbonate (DEPC)-treated distilled water. The RNA content was then quantified with UV spectrophotometer at A_{260} absorbance.

Competitive RT-PCR for GnRH receptor mRNA

Competitive RT-PCR was used as described by

Seong et al.[22] to determine the GnRH-R mRNA levels. The GnRH-R primers were synthesized based on the rat GnRH-R cDNA sequence.[23] The upstream primer was 5'-CTTGAAGCCCGTCCT TGGAGAAAT-3' and the downstream primer was 5'-GCGATCCAGGCTAATCACACCAT-3'. The primers were designed so that the predicted sizes of the PCR products were 441 bp for the native GnRH-R and 280 bp for the mutant GnRH-R, respectively. After linearlization of the plasmids containing both native and mutant GnRH-R cDNA, the native and mutant GnRH-R cRNAs were synthesized by T7 RNA polymerase (Promega, Madison, WI, USA). The concentrations of native and mutant GnRH-R cRNAs were measured with UV spectrophotometer at A_{260}.

The native and mutant cRNA templates were co-reverse transcribed by 200U of RNase H⁻ moloney murine leukemia virus (MMLV) reverse transcriptase (GIBCO-BRL, Grand Island, NY, USA). Subsequently, the PCR reaction mixture containing 50pmol of upstream and downstream primers, and 2.5U of *Taq* DNA polymerase (Promega, Madison, WI, USA) was added. When the native and mutant cRNAs are co-amplified in the same tube, the sequence homology between them may cause hybrids of native and mutant DNA at the annealing temperature.[24] Therefore, PCR amplification was carried out in a two-step procedure to reduce the potential for hybrid formation (first step: denaturation at 94℃ for 1 min, primer annealing at 60℃ for 1 min, and primer extension at 72℃ for 2 min; second step: denaturation at 85℃ for 1 min, primer annealing at 60℃ for 1 min, and primer extension at 72℃ for 2 min). To determine GnRH-R mRNA levels, 26 cycles of the first step and 5 cycles of the second step were used. Five microliter aliquots of the PCR products were electrophoresed on a 2.0% agarose gel in TAE buffer, stained with ethidium bromide and photographed under UV illumination with Polaroid 665 type negative and positive films (Polaroid, Cambridge, MA, USA). The negative film was used for densitometric scanning of native and mutant signals.

RT-PCR for GnRH mRNA

Conventional RT-PCR was performed according

to the method described elsewhere. The PCR primers for GnRH were synthesized based on the sequence of the rat hypothalamic GnRH cDNA.[25] The upper primer was 5'-CACTATGGTCACCA GCGGGG-3' and the lower primer was 5'-AGAG CTCCTCGCAGATCCCTAAG A-3'. The predicted size of the PCR product was 375 bp. The thermoprofile of the PCR was as follows: denaturation at 94℃ for 1 min, primer annealing at 60℃ for 1 min, and primer extension at 72℃ for 2 min.

Data analysis

GnRH-R signals on negative film were measured with a densitometric scanner (Hoefer, San Francisco, CA, USA). The amounts of GnRH-R mRNA were calculated from the native-to-mutant ratio using a standard curve. The quantitative data represent the mean ± SEM of at least four cultures expressed as the percent change as compared with the control samples incubated without hormone treatment. Statistical differences were assessed by the Student's t test for unpaired data, and $p < 0.05$ was considered significant.

RESULTS

Validation of competitive RT-PCR for GnRH-R mRNA in the granulosa cells

For the construction of the standard curve, 100pg of mutant GnRH-R cRNA was co-amplified with a serial dilution of native GnRH-R cRNA (20, 50, 100, 200, 500, 1000 and 2000 pg). The plot of the native-to-mutant signal ratio verses the concentration of native cRNA revealed a linear relationship (Fig. 1). The regression coefficient (γ) of the standard curve was above 0.997. GnRH-R mRNA in the total RNA (0.5 μg) from cultured granulosa cells was competed with 100 pg of mutant GnRH-R cRNA. The GnRH-R mRNA levels were calculated based on the standard curve.

GnRH transcripts in cultured granulosa cells

To determine whether GnRH or GnRH-like peptides are produced in ovarian cells, GnRH
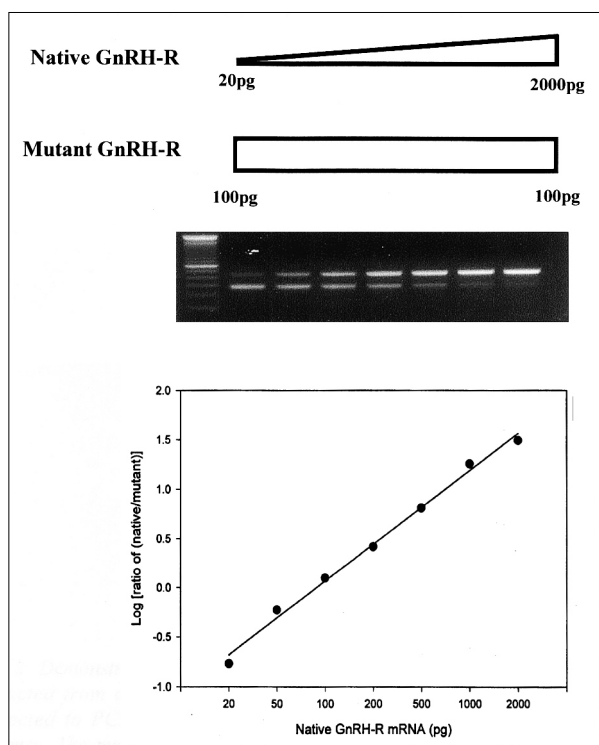
Fig. 1. Standard curve of competitive RT-PCR for quantifying the GnRH-R mRNA levels in cultured granulosa cells. The fixed amount of mutant GnRH-R cRNA (100 pg) was co-amplified with various concentrations of native GnRH-R cRNA (20, 50, 100, 200, 500, 1000, 2000 pg). Each of PCR product was separated on 2.0 % agarose gel and stained with ethidium bromide. A plot of ratios of the native: mutant GnRH-R signals against different amounts of native GnRH-R cRNA revealed a linear relationship.
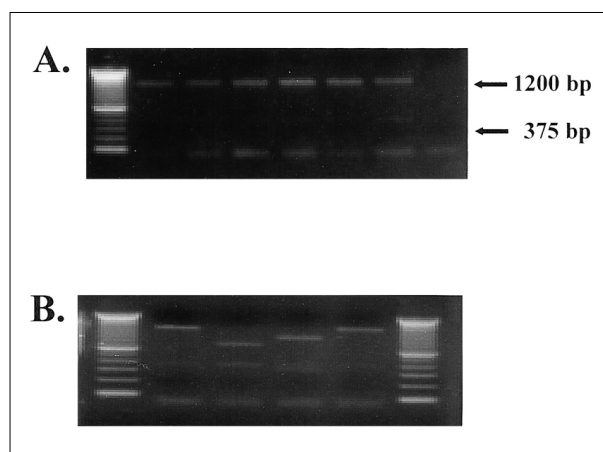

Fig. 2. Demonstration of GnRH mRNA in cultured granulosa cells. A: Total RNA (0.5 μg) extracted from the cultured granulosa cells was reverse-transcribed and aliquots were subjected to PCR amplication. B: The PCR products were treated with restriction enzymes. The major band (1200 bp) was cut into two bands with Hind III (lane 3) and Pst 1 (lane 4), but not with Eco R1 (lane 5). Lane 1, 6: 100 bp marker, Lane 2: control PCR product

transcript expression was examined in the cultured granulosa cells by RT-PCR. The GnRH transcripts detected in the granulosa cells revealed two bands of PCR products. The major band was approximately 1200 bp and the other band was 375 bp, which is a fully processed GnRH gene transcript (Fig. 2A). When the PCR products were treated with Hind III or Pst 1 where the restriction sites reside in intron 1, the major PCR products (1200 bp) were cut into two bands. However, the major bands were not affected by the treatment with Eco R1 which restriction site resides in intron 2, suggesting an inclusion of intron 1 in the major PCR product (Fig. 2B).

GnRH-R mRNA expression in cultured granulosa cells

To determine the time course of GnRH-R

mRNA expression in cultured granulosa cells, the GnRH-R mRNA levels were measured by competitive RT-PCR during the culture period. The GnRH-R mRNA levels were significantly higher after 3 h culture. Thereafter, the levels gradually decreased during the 24 h culture period (Fig. 3). Accordingly, to determine the factors that regulate GnRH-R mRNA expression in the granulosa cells, granulosa cells that were cultured for 3 h were used.

Effect of GnRH on GnRH-R mRNA expression in the granulosa cells

The granulosa cells were exposed to different doses of GnRH for 3 h in order to demonstrate that GnRH-R mRNA expression in the granulosa cells is homologously regulated by GnRH in the same way as in the pituitary gland. Treatment with GnRH did not modulate GnRH-R mRNA expression at any given doses (Fig. 4A). In addition, to evaluate the time course effect of GnRH, the GnRH-R mRNA levels were determined at 0, 3, 6, 9, 12 and 24 h after the treatment with 1 μM GnRH. As seen in Fig. 4B, GnRH-R mRNA expression was not significantly altered by GnRH treatment. Thus, GnRH itself does not homologously regulate GnRH-R mRNA
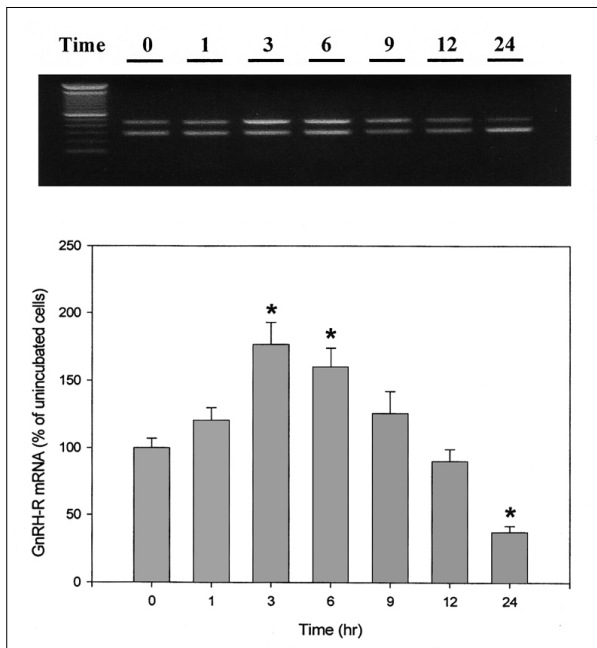
Fig. 3. Time-dependent changes in GnRH-R mRNA levels in cultured granulosa cells without any treatment. Cells were cultured without any treatment for the indicated time. Total RNA samples (0.5 μg) were used for determining the GnRH-R mRNA levels. The GnRH-R mRNA values are expressed as a percentage of the value in unincubated cells (0 h) after a calculation based on the standard curve. Each bar represents the mean ± SEM of repeated experiments (n=5). *p<0.01 (vs 0 h).

Fig. 4. Dose (A) and time (B) effect of GnRH on GnRH-R mRNA expression in cultured granulosa cells. Total RNA samples (0.5 μg) were used for determining the GnRH-R mRNA levels. The GnRH-R mRNA values are expressed as a percentage of the value in untreated cells (A) and unincubated cells at 0 h (B) after a calculation based on the standard curve. Each bar represents the mean ± SEM of repeated experiments (n=4-5).

expression in the granulosa cells. Simultaneous treatment with different doses of GnRH and FSH (50 ng/ml) tended to increase the GnRH-R mRNA levels although the increases were not statistically significant (Fig. 5).

Effect of FSH on GnRH-R mRNA expression in the granulosa cells

The granulosa cells were exposed to different doses of FSH for 3 h in order to determine if FSH modulates GnRH-R mRNA expression in the granulosa cells. GnRH-R mRNA expression were reduced with increasing doses of FSH, showing a significant decrease at 50 ng/ml (p <0.05) and 100 ng/ml (p< 0.01) FSH respectively (Fig. 6A). In addition, GnRH-R mRNA levels were determined at 0, 3, 6, 9, 12 and 24 h after FSH treatment (50 ng/ml) to assess the time course effect. As seen in Fig. 6B, GnRH-R mRNA expression was significantly lower up to 9 h following FSH treatment, and returned to the basal level thereafter.
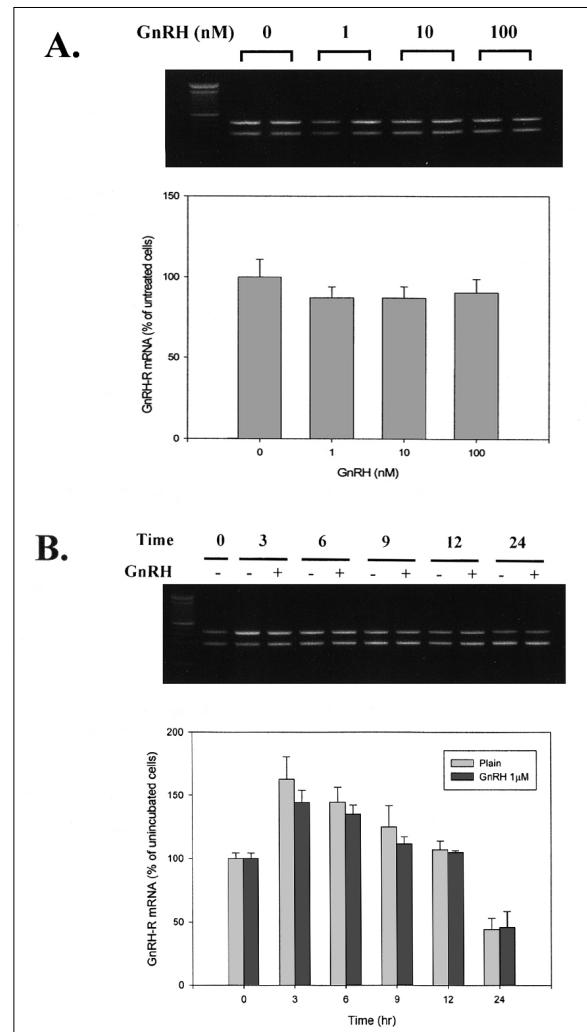
The treatment with forskolin significantly lowered GnRH-R mRNA expression (p <0.01) to the level of GnRH-R mRNA that was suppressed by FSH (Fig. 7).

DISCUSSION

GnRH plays a pivotal role in mammalian reproduction. As a hypothalamic hormone, it controls the synthesis and release of both LH and FSH from the anterior pituitary. Although GnRH
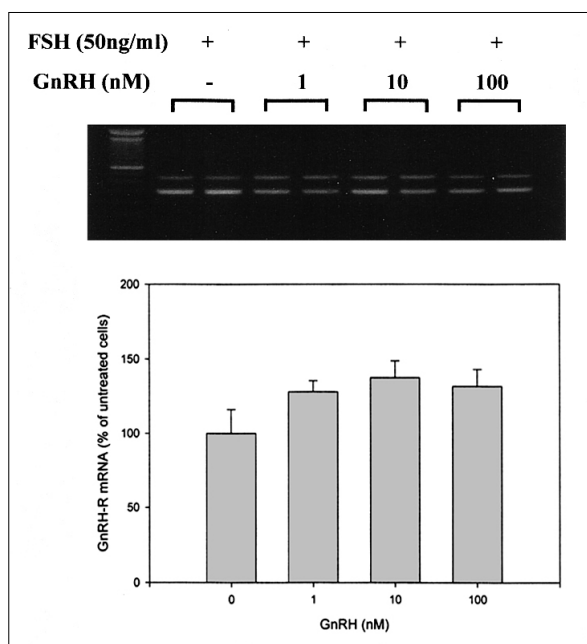
Fig. 5. Effect of different doses of GnRH co-treated with FSH (50 ng/ml) on GnRH-R mRNA expression in cultured granulosa cells. Total RNA samples (0.5 μg) were used for determining the GnRH-R mRNA levels. The GnRH-R mRNA values are expressed as a percentage of the value in untreated cells after a calculation based on the standard curve. Each bar represents the mean ± SEM of repeated experiments (n=4).
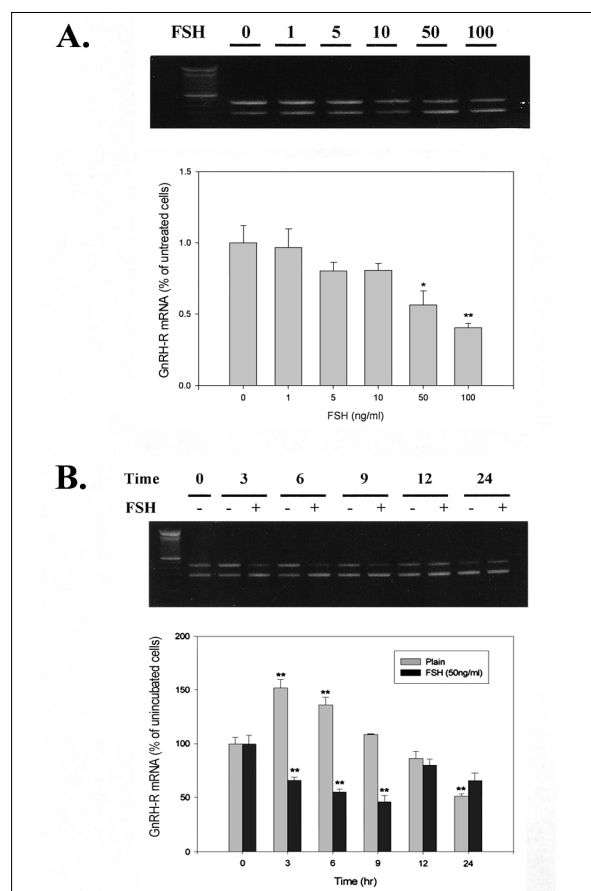

Fig. 6. Dose-dependent (A) and time-dependent (B) effect of FSH on GnRH-R mRNA expression in cultured granulosa cells. Total RNA samples (0.5 μg) were used for determining the GnRH-R mRNA levels. The GnRH-R mRNA values are expressed as a percentage of the value in untreated cells (A) and unincubated cells at 0 h (B) after a calculation based on the standard curve. Each bar represents the mean ± SEM of repeated experiments (n=4). *p < 0.05, **p < 0.01 (vs no FSH or 0 h).

is primarily recognized for its pituitary actions, it has been found to be an important paracrine/autocrine regulator in the ovaries.[26,27]

In the present study, the presence of GnRH transcripts in addition to GnRH-R mRNA expression in the granulosa cells isolated from small antral follicles was demonstrated by competitive RT-PCR. As seen in Fig. 2, the PCR products showed two bands. The major band of the PCR products was about 1.2 kb which might retain intron 1. The minor band was a genuine GnRH transcript identical to the hypothalamic GnRH. In the studies reported by Okikawa et al.[6] and Goubau et al.,[7] GnRH transcripts were found in the granulosa cells. The former reported the major GnRH transcript as a genuine GnRH transcript found in the hypothalamus. The latter reported that the major GnRH transcript retained intronic sequences while the minor GnRH transcript was a genuine GnRH transcript found in the hypothalamus. Our findings are consistent with Goubau et al.[7] However, the major GnRH transcripts detected by Goubau et al.[7] consist of

exon 2, 3, 4, intron 2 and 3 plus an undefined sequence, presumably short sequence of the first intron, whereas the major GnRH transcripts in our study consist of exon 1, 2, 3 and intron 1 sequences. The discrepancy between these findings and those reported by Goubau et al.[7] may be due to a difference in the PCR primer design. Recently, Seong et al.[28] showed that the GnRH primary transcripts were more prevalent than mature GnRH mRNA in non-GnRH producing tissues, and the extremely low level of mature GnRH mRNA, as compared with its premature GnRH transcripts, is most likely due to the intrinrsic weakness of the intron 1 splice sites. Therefore, our results together with others suggest
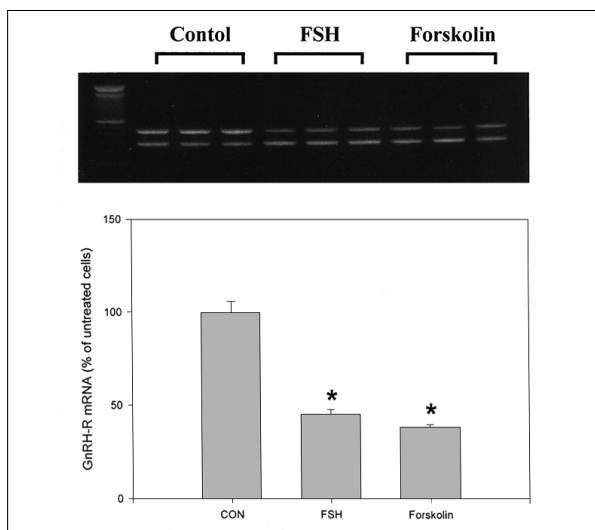
Fig. 7. Effects of FSH (50 ng/ml) and forskolin (100 μM) on GnRH-R mRNA expression in cultured granulosa cells. Total RNA samples (0.5 μg) were used for determining the GnRH-R mRNA levels. The GnRH-R mRNA values are expressed as a percentage of the value in untreated cells (control) after a calculation based on the standard curve. Each bar represents the mean $\pm$ SEM of repeated experiments (n=4). *p < 0.01 (vs contorl).

that GnRH transcripts are present in the granulosa cells and GnRH transcripts may retain intron 1, which was not spliced because of the intrinsic weakness of the intron 1 splice site.

In examining whether GnRH-R mRNA is expressed in the granulosa cells isolated from the small antral follicles, it was shown that GnRH-R mRNA expression was increased abruptly within 3 h culture, and decreasing thereafter. The reason why GnRH-R mRNA expression in the granulosa cells was changed spontaneously in this way is not as yet understood. However, it is assumed that the granulosa cells in culture without treatment may rapidly go through an apoptotic process. A time-dependent, spontaneous onset of internucleosomal DNA fragmentation, which is a characteristic of apoptotic cell death, has been reported to occur in granulosa cells during culture under serum-free conditions.[29] Furthermore, the treatment of hypophysectomized DES-treated rats with a GnRH agonist directly increased internucleosomal DNA fragmentation in the ovaries in a time- and dose-dependent manner.[13,30]

It is well documented that the GnRH-R level is both up- and down-regulated by GnRH in the pituitary gland.[15,17,18] The effects of GnRH on

GnRH-R mRNA expression in the granulosa cells seem to be diverse and dependent on the state of follicular maturation. Recent reports have indicated that GnRH in a certain concentration range up-regulates GnRH-R mRNA in the granulosa cells of preovulatory follicles.[8,19] Their results appear to be supported by the fact that GnRH induces oocyte maturation[10] and follicular rupture in the preovulatory follicles.[11] On the contrary, a number of studies have implicated a role of GnRH in the progression of follicular atresia.[13,14] However, Kogo et al.[30] revealed that the treatment of hypophysectomized immature rats with GnRH agonist markedly increased apoptotic cell death rate of the granulosa cells but GnRH-R mRNA expression was unaffected. In our experiment, GnRH itself did not modulate GnRH-R mRNA expression in granulosa cells isolated from the small antral follicles of immature rats. Rather, GnRH-R mRNA expression was down-regulated by FSH in a dose- and time-dependent manner.

The effect of FSH on GnRH-R mRNA expression in the granulosa cells appears to be diverse and somewhat controversial depending on the stage of follicular development. Olofsson et al.[19] reported that FSH showed no effect on GnRH-R mRNA expression in cultured granulosa cells isolated from preovulatory follicles. However, in atretic follicles, GnRH-R mRNA expression was significantly higher at 18:00 h on proestrus and again on the morning of estrus when the FSH levels were elevated.[31] Since FSH receptor mRNA is rarely expressed in the atretic follicles, it is unlikely that FSH directly regulates GnRH-R mRNA expression in the granulosa cells of the atretic follicles. Therefore, they suggested that the action of FSH on healthy follicles resulted in the release of an unknown factor(s) that acted in a paracrine manner to regulate GnRH-R mRNA expression in the atretic follicles nearby. In our experiment, FSH decreases GnRH-R mRNA in the granulosa cells isolated from small antral follicles. This result might partially explain the mechanism by which FSH exerts anti-atretic effects on the granulosa cells since GnRH has been well known to have atretic effects on granulosa cells. When treated with forskolin, an adenylate cyclase activator, GnRH-R mRNA expression decreased to the level that was similar to the level induced by

FSH. This suggests that a cAMP dependent pathway may mediate the action of FSH in decreasing GnRH-R mRNA expression. Indeed, Alarid et al.[32] found that forskolin induced a time-dependent decline in GnRH-R mRNA levels in αT3-1 cells.

In summary, GnRH transcripts were detected in the granulosa cells isolated from small antral follicles and major GnRH transcript contains intronic sequences. GnRH does not homologously regulate GnRH-R mRNA expression in cultured granulosa cells. Rather, FSH may negatively regulate GnRH-R mRNA expression in the granulosa cells through a cAMP-protein kinase A pathway.

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