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Homologous Upregulation of GnRH Receptor mRNA by Continuous GnRH in Cultured Rat Pituitary Cells

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The present study examined the effects of continuous treatment with gonadotropin-releasing hormone (GnRH) on GnRH receptor (GnRH-R) mRNA levels in dispersed cultures of rat pituitary cells. Pituitary GnRH-R mRNA levels were determined by competitive reverse transcriptase polymerase chain reaction. When pituitary cells were continuously exposed to a low dose of GnRH (0.2 n*M*), GnRH-R mRNA levels were transiently increased. The levels of GnRH-R mRNA were significantly increased up to 6 h and diminished to untreated levels by 24 h. Luteinizing hormone (LH) release was also increased significantly up to 12 h, maintaining similar levels in LH release thereafter. When GnRH antagonist ([D-pGlu[1], D-Phe[2], D-Trp[3,6]]-LH-RH) was added to the cultures together with GnRH (0.2 n*M*) for 6 h, the stimulatory effect of GnRH on GnRH-R mRNA levels and LH release was significantly diminished in a dose-related manner. In another experiment, pituitary cells were treated with various doses of GnRH (0.02–200 n*M*) for a relatively short (6 h) or a longer (24 h) period. When pituitary cells were exposed for 6 h, all doses of GnRH (0.02–200 n*M*) significantly increased GnRH-R mRNA levels in a dose-dependent manner. By contrast, continuous exposure to GnRH for 24 h was ineffective in changing pituitary GnRH-R mRNA levels at any given doses. These results indicate that the duration of GnRH treatment is critical for upregulation of GnRH-R mRNA by continuous GnRH. When pituitary cells were treated for 6 h with either a continuous mode of GnRH (0.2 n*M*) or an hourly pulsatile mode of GnRH (0.2 n*M*, 6 min/h), both treatments significantly augmented GnRH-R mRNA levels. Thus, the modes of GnRH application, if treated for a relatively short period, do not appear to make a significant difference in upregulation of GnRH-R mRNA levels. Collectively, our data provide strong evidence that continuous GnRH application is able to upregulate pituitary GnRH-R mRNA levels, if treated for a relatively short period (6 h).

Key Words: Gonadotropin-releasing hormone receptor; gonadotropin-releasing hormone; anterior pituitary; gene expression; primary cultures; rat.

Introduction

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in regulating the reproductive functions. GnRH is released into the hypothalamo-hypophyseal portal vein and binds to GnRH receptor (GnRH-R) on pituitary gonadotropes to regulate the synthesis and secretion of gonadotropins *(1,2)*. It has been known that GnRH-R is regulated by GnRH itself. A pulsatile administration of GnRH increased the number of GnRH-R in pituitary *(3)*. However, when pituitary cells were continuously treated with physiological doses of GnRH (10^{-11}–10^{-9} *M*), the number of GnRH-R was initially downregulated and subsequently upregulated *(4–6)*. In addition, continuous treatment with high doses of GnRH decreased the number of GnRH-R *(7)*. Thus, the effect of continuous GnRH on the regulation of the number of GnRH-R appears to depend on the duration of GnRH treatment and concentration of GnRH. Recent cloning of GnRH-R cDNA *(8,9)* has allowed the investigation of GnRH-R gene regulation. However, little evidence is available on the homologous regulation of GnRH-R gene expression in primary cultures of pituitary cells *(10–12)*. Kaiser et al. *(10)* found that a pulsatile administration of GnRH for 24 h increased pituitary GnRH-R mRNA levels, whereas a continuous infusion of GnRH for 24 h failed to alter pituitary GnRH-R mRNA levels, concluding that continuous application of GnRH does not affect pituitary GnRH-R gene expression. However, the effects of concentration and treatment duration of continuous GnRH on GnRH-R mRNA levels were not extensively examined in previous studies. Therefore, the present study investigated whether the effect of a continuous mode of GnRH on GnRH-R mRNA levels depends on treatment

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duration and concentration of GnRH in dispersed cultures of rat pituitary cells. To determine pituitary GnRH-R mRNA levels precisely, competitive reverse transcriptase polymerase chain reaction (RT-PCR) was employed.

Results

Validation of Competitive RT-PCR for GnRH-R mRNA Levels

For the construction of the standard curve, 100 fg of mutant GnRH-R cRNA were coamplified with a serial dilution of native GnRH-R cRNA (10, 25, 50, 100, 250, 500, 1000, and 2500 fg). The plot for ratios of native-to-mutant signals vs concentrations of native cRNA revealed a linear relationship (Fig. 1). The regression coefficient (*r*) of the standard curve was above 0.950. GnRH-R RNA in pituitary total RNA (0.5 μg) competed with 100 fg of mutant GnRH-R cRNA. GnRH-R mRNA levels were calculated based on the standard curve.

Effect of Continuous GnRH on GnRH-R mRNA Levels and Luteinizing Hormone Release

To elucidate whether continuous GnRH application modulates GnRH-R gene expression, dispersed cultures of rat pituitary cells were treated with a low dose of GnRH (0.2 n*M*). The concentration of luteinizing hormone (LH) secreted into medium and GnRH-R mRNA was measured at 3, 6, 12, and 24 h after GnRH treatment. The levels of GnRH-R mRNA were increased transiently up to 6 h and reduced to untreated basal levels by 24 h after treatment with GnRH (Fig. 2). LH release was significantly increased up to 12 h, and the increment of LH release was not noticeable thereafter (Fig. 3). In the next experiment, to verify whether this stimulatory effect of continuous GnRH on its own receptor gene expression is specific, GnRH antagonist ([D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LH-RH) was added to the cultures together with GnRH. Based on the time course effect of continuous GnRH application on GnRH-R mRNA levels, GnRH and GnRH antagonist were simultaneously treated for 6 h. When pituitary cells were treated with GnRH alone, GnRH-R mRNA levels as well as LH release were evidently increased (Fig. 4). However, the application of GnRH antagonist reduced the GnRH-induced increase in GnRH-R mRNA levels and LH release in a dose-related manner, although the GnRH-stimulated LH release was, in comparison with GnRH-R mRNA levels, less responsive to 2 n*M* GnRH antagonist. To determine whether the concentration of GnRH is also critical for the regulation of GnRH-R mRNA levels, pituitary cells were continuously exposed to various doses of GnRH (0.02–200 n*M*) for a relatively short (6 h) or a longer (24 h) period. All doses of GnRH (0.02–200 n*M*) treated for 6 h significantly increased GnRH-R mRNA levels in a dose-dependent manner, reaching a maximum with 2 n*M* GnRH (Fig. 5A). LH release was also increased in a dose-related pattern (Fig. 5B). When

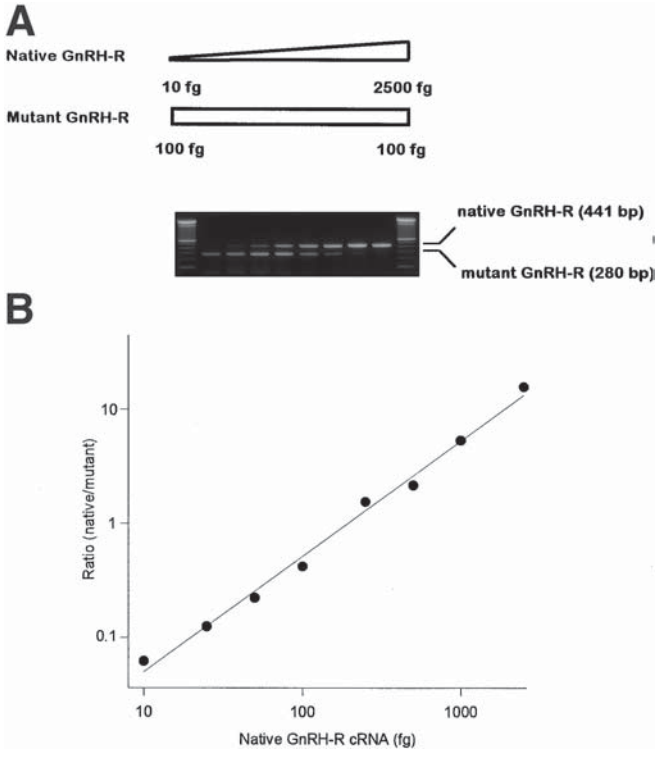

Fig. 1. Standard curve of competitive RT-PCR for quantitation of GnRH-R mRNA levels. **(A)** A constant amount of mutant GnRH-R cRNA was coamplified with various concentrations of native GnRH-R cRNA. Each PCR product was separated on a 2.0% agarose gel. **(B)** Plot of ratios of native/mutant GnRH-R signals against different amounts of native GnRH-R cRNA revealed a linear relationship. Mutant GnRH-R cRNA (100 fg) was coamplified with 10, 25, 50, 100, 250, 500, 1000, and 2500 fg of native GnRH-R cRNA.

pituitary cells were continuously exposed to GnRH for 24 h, however, the levels of GnRH-R mRNA were not altered by any given doses of GnRH (Fig. 6).

Effect of GnRH Administration Mode (Pulsatile vs Continuous) on GnRH-R mRNA Levels

We further examined whether the modes of GnRH application (pulsatile vs continuous) differentially influence GnRH-R gene expression during a relatively short period (6 h). Cultured pituitary cells were treated with pulsatile GnRH (0.2 n*M*, 6 min/h) for 6 h or with continuous mode of GnRH (0.2 n*M*) for 6 h. Both modes of GnRH application significantly augmented GnRH-R mRNA levels, and the increment of GnRH-R mRNA levels by continuous mode of GnRH was slightly greater than that by pulsatile mode of GnRH (Fig. 7).

Discussion

The present study showed that continuous GnRH application transiently increased GnRH-R mRNA levels as effectively as pulsatile administration of GnRH in cultured rat pituitary cells. GnRH-R mRNA levels were

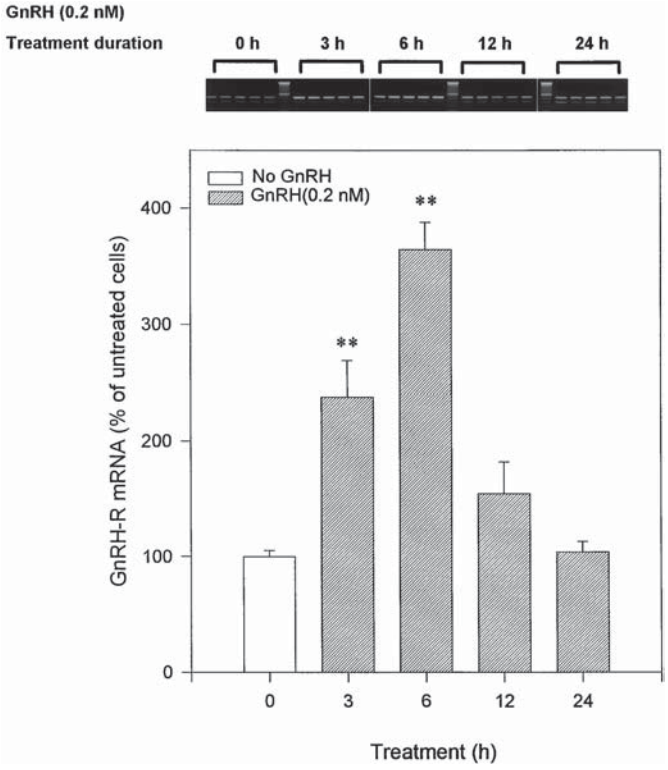

Fig. 2. Time course changes in GnRH-R mRNA in dispersed cultures of rat pituitary cells. Cells were treated continuously with GnRH (0.2 n*M*) for the indicated durations. Total RNA samples (0.5 μg) were used for determination of GnRH-R mRNA levels. The values of GnRH-R mRNA are expressed as a percentage of the levels in untreated cells at 0 h after calculation based on the standard curve. **(Top)** PCR products separated on a 2.0% agarose gel and stained with ethidium bromide. Each bar represents the mean ± SEM. (n = 10–12 from three experiments). □, No GnRH; ■, GnRH (0.2 n*M*); $^{**}p < 0.01$ (vs no GnRH).

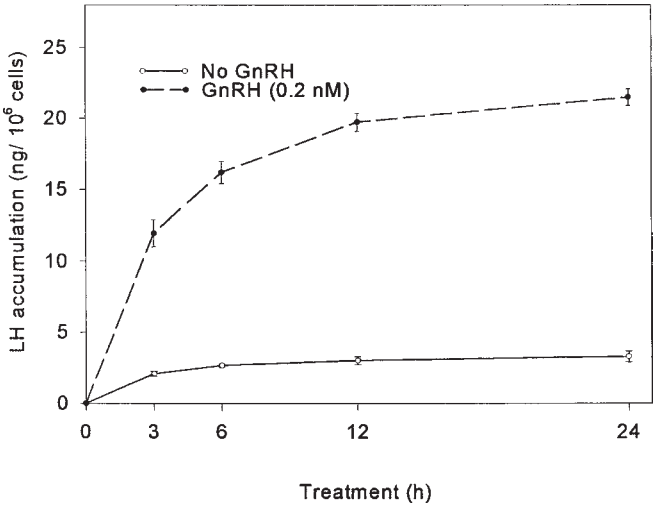

Fig. 3. Time course changes in LH accumulation in dispersed cultures of rat pituitary cells. Cells were treated continuously with GnRH (0.2 n*M*) for the indicated durations. Each bar represents the mean ± SEM (n = 12 from three experiments). (○—○), No GnRH; (● – – ●), GnRH (0.2 n*M*).

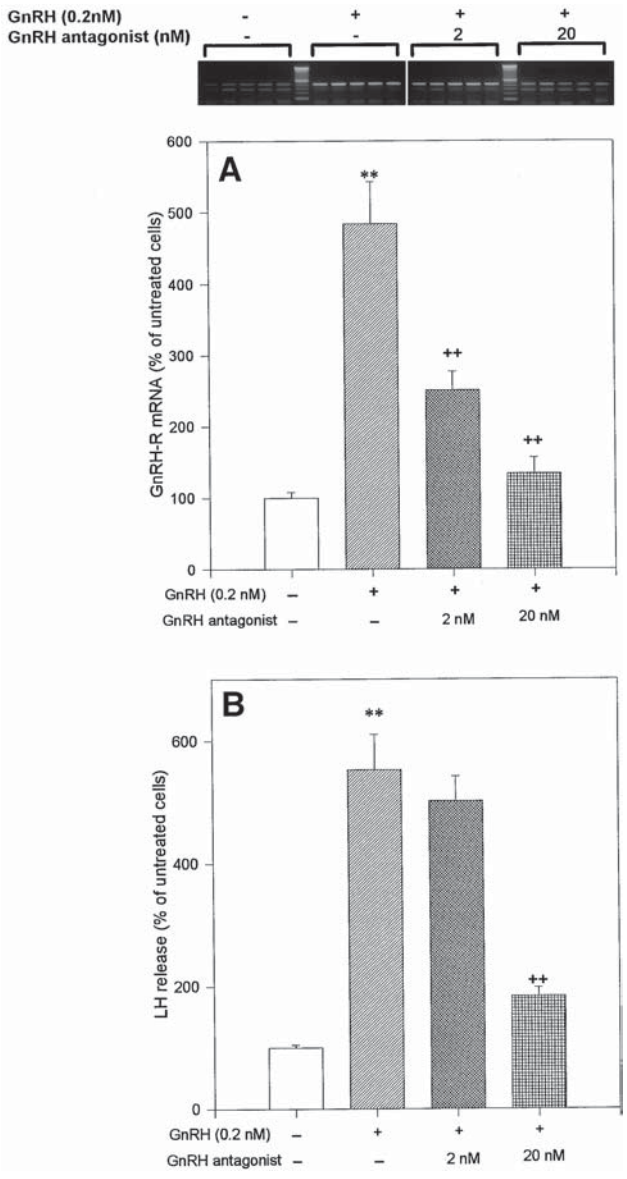

Fig. 4. The effect of GnRH antagonist (2 or 20 n*M*) on GnRH-induced GnRH-R mRNA **(A)** and LH release **(B)** in dispersed cultures of rat pituitary cells. Cells were treated continuously with GnRH (0.2 n*M*) and GnRH antagonist ([D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LH-RH) for 6 h. The values of GnRH-R mRNA are expressed as a percentage of the levels in untreated cells at 6 h. Each response of LH release is expressed as a percentage of LH release from untreated cells at 6 h. **(Top)** PCR products separated on a 2.0% agarose gel and stained with ethidium bromide. Each bar represents the mean ± SEM. (n = 12 from three experiments). $^{**}p < 0.01$ (vs no GnRH); $^{++}p < 0.01$ (vs GnRH alone).

increased significantly up to the initial 6 h and returned to untreated basal levels by 24 h. This homologous upregulation of GnRH-R mRNA by continuous GnRH application for a relatively short period (6 h) appears to be specific since GnRH antagonist evidently prevented a GnRH-induced increase in pituitary GnRH-R mRNA levels. A short period (6 h) of exposure to various doses of GnRH (0.02–200 n*M*) increased GnRH-R mRNA levels signifi-

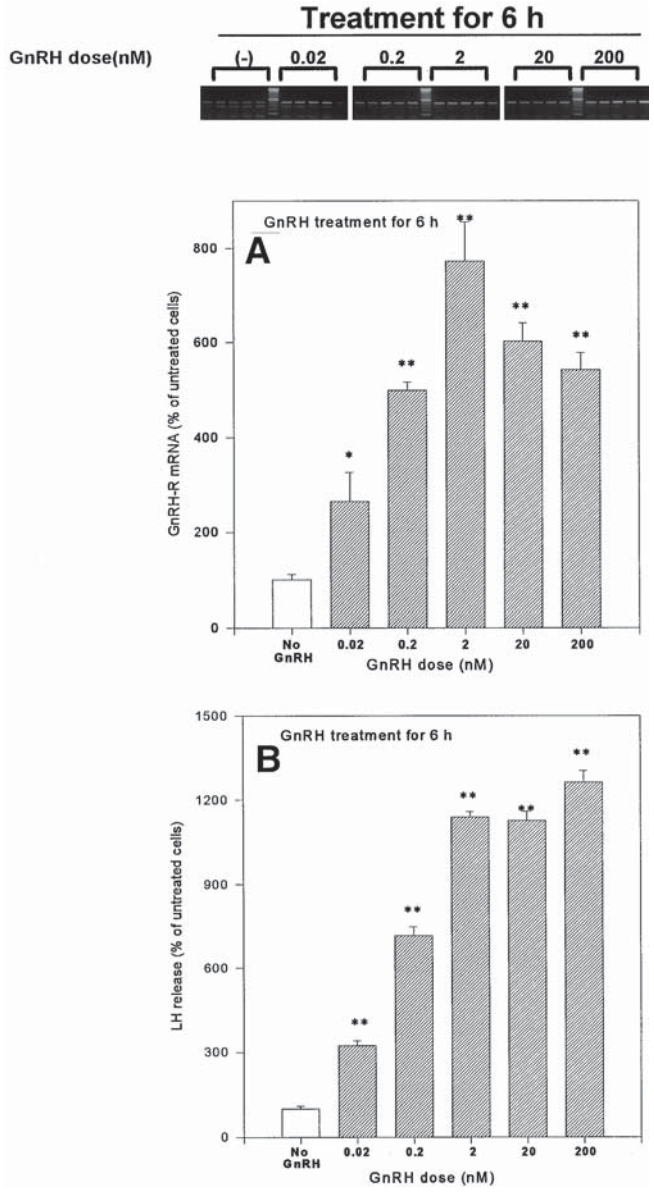

Fig. 5. Dose effect of GnRH at 6 h on GnRH-R mRNA **(A)** and LH release **(B)** in dispersed cultures of rat pituitary cells. Cells were treated continuously with various doses of GnRH for 6 h. The values of GnRH-R mRNA are expressed as a percentage of the levels in untreated cells at 6 h. Each response of LH release is expressed as a percentage of LH release from untreated cells at 6 h. **(Top)** PCR products separated on a 2.0% agarose gel and stained with ethidium bromide. Each bar represents the mean ± SEM. (n = 10–12 from three experiments). *$p < 0.05$ (vs no GnRH); **$p < 0.01$ (vs no GnRH).

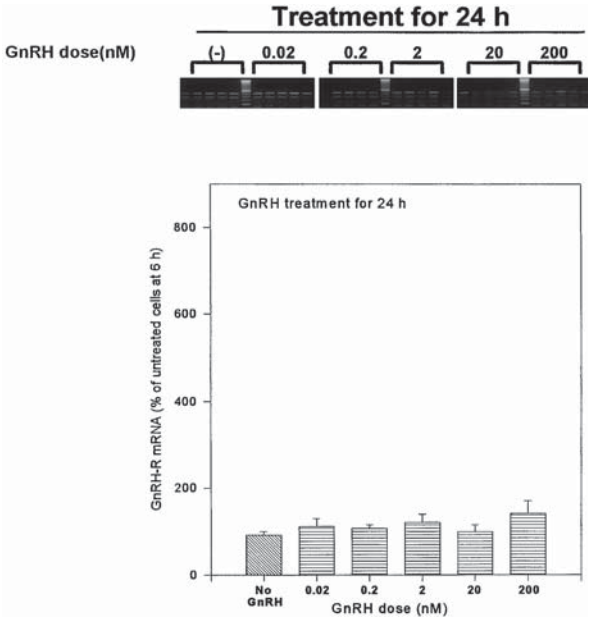

Fig. 6. Dose effect of GnRH at 24 h on GnRH-R mRNA in dispersed cultures of rat pituitary cells. Cells were treated continuously with various doses of GnRH for 24 h. The values of GnRH-R mRNA are expressed as a percentage of the levels in untreated cells at 6 h. **(Top)** PCR products separated on a 2.0% agarose gel and stained with ethidium bromide. Each bar represents the mean ± SEM. (n = 11 to 12 from three experiments).

cantly. These results provide strong evidence that even continuous application of GnRH is able to upregulate pituitary GnRH-R mRNA levels, if treated for a relatively short period (6 h).

It has been known that the responsiveness of gonadotropes is desensitized by continuous GnRH, but not by pulsatile GnRH *(13)*. However, evidence has been accumulated that the expression of the glycoprotein α-subunit gene was increased after continuous exposure to GnRH *(14–18)*, suggesting the possibility for continuous GnRH to upregulate responses in gonadotropes. Indeed, recently Lin and Conn *(19)* demonstrated that continuous GnRH administration increased GnRH-R gene expression. In their study, short-term GnRH administration significantly stimulated the luciferase reporter gene activity in GGH3 cells transfected with GnRH-R-Luc vector (vector containing luciferase reporter gene and promoter fragment of mouse GnRH-R gene). The present study, together with our previous data *(20)*, is quite consistent with the findings of Lin and Conn *(19)*.

Exposure of gonadotropes to GnRH for a longer period (24 h) failed to alter GnRH-R mRNA levels at any given doses. These data were consistent with the previous study by Kaiser et al. *(10)* in which continuous delivery of GnRH (10 n*M*) for 24 h was ineffective in increasing GnRH-R mRNA levels in superfused primary monolayer cultures of rat pituitary cells. Thus, continuous GnRH application for 24 h appears to desensitize GnRH-R gene expression. Little evidence is available on the mechanisms underlying GnRH-induced desensitization. However, recent work has demonstrated that GnRH action is mediated by mitogen-activated protein kinase (MAPK), and pulsatile GnRH was necessary to maintain MAPK activity *(21)*. Whereas continuous GnRH treatment increased MAPK activity only for the initial 2 h and returned to baseline thereafter, pulsatile

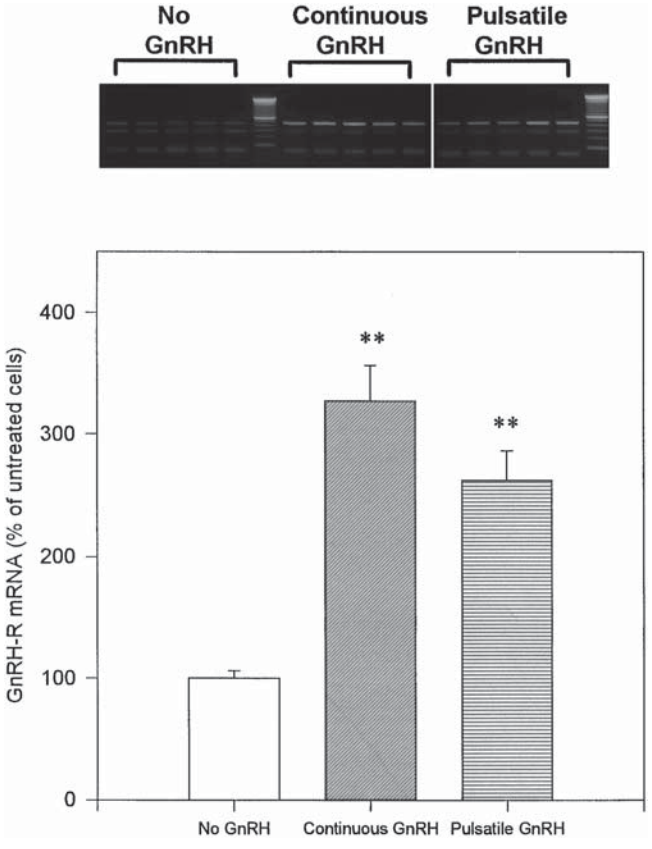

Fig. 7. The changes of GnRH-R mRNA levels by continuous or pulsatile GnRH in dispersed cultures of rat pituitary cells. Cells were treated continuously with GnRH (0.2 n*M*), or hourly with GnRH (0.2 n*M*, 6 min/h) for 6 h. The values of GnRH-R mRNA are expressed as a percentage of the levels in untreated cells at 6 h. (**Top**) PCR products separated on a 2.0% agarose gel and stained with ethidium bromide. Each bar represents the mean ± SEM ($n = 8$ from two experiments). $^{**}p < 0.01$ (vs no GnRH).

GnRH stimulated MAPK activity throughout the examined duration of 8 h *(21)*. This study suggests that a decrease in MAPK activity might be responsible for desensitizing the response of gonadotropes to continuous GnRH, although the mechanism by which continuous GnRH decreases MAPK activity has not been clearly understood.

Our results showed that GnRH-R mRNA levels from 6 to 12 h of GnRH stimulation abruptly dropped. In the meantime, we are not able to explain clearly this decline within 6 h. An abrupt drop of GnRH-R mRNA levels from 6 to 12 h of GnRH administration might be owing to an acceleration of basal turnover rate of preexisting GnRH-R mRNA or a decrease in transcriptional rate of GnRH-R gene, or both. Kaiser et al. *(10)* reported a similar precipitous drop of GnRH-R mRNA levels from 8 to 12 h of pulsatile GnRH administration. Recently, Lin and Conn *(19)* showed that a GnRH-induced increase in transcriptional activity of mouse GnRH-R gene declined at 12 h of GnRH stimulation, suggesting a drop in transcriptional rate of GnRH-R gene at 12 h. Li et al. *(22)* estimated the basal half-life of GnRH-R mRNA to be 27 h in GnRH-unstimulated GT1-7 neuronal cells. The precipitous drop of GnRH-R mRNA levels at 12 h could not be explained by this long basal turnover rate of GnRH-R mRNA and the drop in transcriptional rate of GnRH-R gene. However, the basal turnover rate of LHβ mRNA in GnRH-prestimulated cells in our previous study *(23)* was accelerated, compared with the basal turnover rate of the mRNA in GnRH-unstimulated cells *(24)*. Thus, to explain clearly the precipitous drop of GnRH-R mRNA from 6 to 12 h of GnRH stimulation, further studies on turnover rate of GnRH-R mRNA are required in GnRH-prestimulated cells as well as GnRH-unstimulated cells.

Our results also revealed that continuous GnRH application significantly increased pituitary GnRH-R mRNA levels up to the initial 6 h. Although a change in the number of GnRH-R was not determined in the present study, several previous studies demonstrated that continuous GnRH administration increased the number of GnRH-R in primary cultures of rat pituitary cells *(4–7)*. Continuous GnRH administration decreased the number of GnRH-R initially (1–4 h) and increased it subsequently (6–10 h) *(4–6)*. Therefore, it is conceivable that a GnRH-induced increase in the number of GnRH-R followed upregulation of GnRH-R mRNA levels. By contrast, in the mouse clonal gonadotrope cell line αT3-1 that is arrested in an early developmental stage, no homologous upregulation of GnRH-R mRNA was observed. The modulation of the number of GnRH-R by GnRH is, however, accompanied by changes in the translational activity of GnRH-R mRNA or the distribution of polyribosome-associated GnRH-R mRNA *(25,26)*. These studies suggest that in αT3-1 cells, homologous upregulation of GnRH-R occurs at the translational level rather than at the transcriptional level.

In recent in vivo studies, pituitary GnRH-R mRNA levels were increased by infusion of GnRH in rats and sheep *(27,28)*, and GnRH antagonist reduced GnRH-R mRNA levels in the pituitary of sheep *(29)*. Moreover, a withdrawal of endogenous GnRH by passive immunization with anti-GnRH sera decreased pituitary GnRH-R mRNA levels in sheep *(30,31)*. The inhibition of hypothalamic GnRH input by pentobarbital also reduced pituitary GnRH-R mRNA levels in rats *(11)*. These in vivo studies suggest that hypothalamic GnRH input may be indispensable for the induction of GnRH-R gene expression in the pituitary. Therefore, our in vitro data that GnRH directly regulates GnRH-R gene expression are in good agreement with these in vivo studies.

Interestingly, it has been reported that during the preovulatory LH surge in sheep, the pattern of GnRH secretion may be in a continuous manner *(32)*, although during the majority of the estrous cycle, GnRH is secreted as discrete pulses. Moreover, Nishihara et al. *(33)* demonstrated that the GnRH pulse generator may not be involved in inducing the LH surge in rats. Therefore, it is presumed that GnRH

might be released in a continuous mode during the LH surge in rats. Furthermore, Bauer-Dantoin et al. *(34)* found that pituitary GnRH-R mRNA levels in rats remained elevated throughout the LH surge. Although the present in vitro data cannot be directly extrapolated to in vivo status, upregulation of GnRH-R mRNA expression by continuous GnRH in the present study suggests the possibility that continuous mode of GnRH during the preovulatory LH surge may play a role in regulating pituitary GnRH-R gene expression in vivo.

In summary, we have demonstrated that even continuous GnRH application is able to upregulate transiently pituitary GnRH-R mRNA levels. The molecular mechanism by which GnRH augments GnRH-R mRNA levels is currently under investigation.

Materials and Methods

Preparation of Anterior Pituitary Cell Cultures

Pituitary glands from female Sprague-Dawley rats (150–200 g; provided by Yuhan Research Center, Korea) at random stages of the estrous cycle were used for the preparation of cell cultures, since there was no significant difference in the GnRH-induced increase in GnRH-R mRNA levels in primary cultures between pituitary glands from estrous rats and those from random cycling rats. Anterior pituitary cells were prepared by enzymatic dispersion, with modifications to previously described methods *(35)*. Following decapitation, the anterior pituitary glands were removed and washed in Spinner's Minimal Essential Medium (S-MEM) (Gibco-BRL, Gaithersburg, MD) containing 0.3% bovine serum albumin (BSA) (fraction V) (Sigma, St. Louis, MO) and 10 m*M* HEPES (Sigma). Anterior lobes were cut into several pieces in S-MEM/BSA and then enzymatically digested with 20 mL of S-MEM/BSA containing 0.25% trypsin (1:250) (Difco, Detroit, MI) and DNase (10 μg/gland) (Sigma) for 1 h at 4°C and a further 30 min at 37°C.

Dispersion was facilitated by repeated aspiration and expulsion of the tissue fragments with a fire-polished Pasteur pipet. Dispersed cells were then centrifuged at 400*g* for 10 min. The pellet was resuspended in 20 mL of S-MEM/BSA containing trypsin inhibitor (5 μg/mL) (Sigma) and filtered through lens paper to remove residual tissue fragments. The cell suspension was briefly centrifuged, and the cell pellet was resuspended in α-Minimal Essential Medium (α-MEM) (Gibco-BRL) containing 2.5% fetal bovine serum (Gibco-BRL), 10% horse serum (Gibco-BRL) and antibiotics (100 U of penicillin and 100 mg of streptomycin/mL (Sigma). All sera were dextran-charcoal stripped for the removal of residual steroids. The cells were more than 95% viable, as measured by trypan blue exclusion. Aliquots of these cells (1×10^6 cells/mL) were incubated in multiwell culture plates (Falcon) in 5% CO_2/air at 37°C. Following preincubation, the cells were washed twice with Dulbecco's phosphate-buffered saline (Sigma) to remove serum and nonadherent cells, and thereafter they were further incubated in serum-free medium (α-MEM) containing 0.3% BSA for experiments.

Total RNA Extraction

Total cytoplasmic RNA from the pituitary was extracted by the acid guanidinium thiocyanate-phenol-chloroform method *(36)*. Briefly, 600 μL of denaturing solution containing 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine, and 0.1 *M* 2-mercaptoethanol were added to each well and collected in a microcentrifuge tube. Sixty microliters of 2 *M* sodium acetate (pH 4.0), 600 μL of water-saturated phenol, and 120 μL of chloroform:isoamyl alcohol mixture (49:1) were added. After cooling on ice for 15 min, samples were centrifuged (10,000*g*) at 4°C for 20 min and precipitated with ethanol. After washing with 75% ethanol, the RNA pellet was dried under a vacuum and dissolved in 20 μL of sterilized distilled water. RNA content was then quantified at A_{260} absorbance.

Competitive RT-PCR

Competitive RT-PCR to determine GnRH-R mRNA levels was performed as previously described *(37)*. GnRH-R primers were synthesized based on the sequence of the rat GnRH-R cDNA *(38)*. The upstream primer is 5'-CTTGAAGCCCGTCCTTGGAGAAAT-3', and the downstream primer is 5'-GCGATCCAGGCTAATCACCACCAT-3'. Primers were designed such that the predicted sizes of PCR products were 441 and 280 bp for native and mutant GnRH-R, respectively. After linearization of plasmids containing native and mutant GnRH-R cDNA, native and mutant GnRH-R cRNAs were synthesized by T7 RNA polymerase using an in vitro transcription system kit purchased from Promega (Madison, WI). Concentrations of native and mutant GnRH-R cRNAs were measured with an ultraviolet (UV) spectrophotometer at A_{260}.

Briefly, native and mutant cRNA templates were co-reverse transcribed by 200 U of $RNaseH^-$ moloney murine leukemia virus RT (Gibco-BRL). Subsequently, the PCR reaction mixture containing 50 pmol of up- and downstream primers and 2.5 U of *Taq* DNA polymerase (Promega) was added. When native and mutant cRNAs are coamplified in the same tube, the sequence homology between them may cause hybrids of native and mutant DNA at the annealing temperature *(39)*. Therefore, PCR amplification was carried out with a two-step procedure to reduce hybrids (step 1: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min; step 2: denaturation at 85°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min). To determine GnRH-R mRNA levels, 33 cycles of step 1, and 6 cycles of step 2 were used. Ten-microliter aliquots of 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). Negative film was used for densitometric scanning of native and mutant signals.

Radioimmunoassay of LH

LH was measured by a double-antibody radioimmunoassay using reagents kindly provided by the National Pituitary Agency/NIDDK. Tracer (rLH-I-9) was iodinated by chloramine T. Radiolabeled tracer (^{125}I-rLH) was separated by gel permeation chromatography with Sephadex G-50. The antisera (rLH-S-10) and the reference preparations (rLH-RP-2) were dissolved in 0.01 *M* phosphate buffer containing 0.1% (w/v) BSA. The antisera were diluted from the stock solutions (1:18.75), so that the final tube dilution was 1:135,000. To measure LH concentration, radiolabeled tracer (10,000–15,000 cpm/100 μL), antisera (100 μL; 1:45,000), and unknown samples (100 μL) were incubated for 3 h at 37°C. The antigen-antibody complex was precipitated by adding 0.1 mL of EDTA, 0.1 mL of normal rabbit serum (2%), 0.1 mL of antirabbit IgG (Sigma) and 0.6 mL of polyethyleneglycol (mol wt 8000). After centrifugation at 3000*g* for 20 min, the supernatant was discarded, and the radioactivity present in the precipitate was counted in a gamma counter (Packard, Meriden, CT). Assay sensitivity was 0.1 ng/mL. The intra- and interassay coefficients of variation were 6.5% and 9.8%, respectively.

Data Analysis

GnRH-R signals on negative film were measured with a densitometric scanner (Hoefer, San Francisco, CA). The amounts of GnRH-R mRNA were calculated from the native-to-mutant ratio using a standard curve. Data were statistically evaluated using the one-way analysis of variance, followed by Fisher's least significant difference test for a post-hoc comparison. When appropriate, data were evaluated using the Student *t*-test. All values are given as the means ± SE. Statistical significance was set at $p < 0.05$.

Acknowledgments

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