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Effect of Growth Hormone Releasing Hormone on the Proliferation of Cultured Cells Derived from Rat Anterior Pituitary Gland

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배양중인 흰쥐 뇌하수체 전엽 세포의 증식에 미치는 Growth Hormone Releasing Hormone (GHRH)의 영향

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ABSTRACT: Growth hormone releasing hormone (GHRH), the major hypothalamic stimulus of GH secretion from the anterior pituitary gland, has been found to be present in several extrahypothalamic sites including placenta, testis, ovary and anterior pituitary gland. The present study was performed to elucidate the role of pituitary GHRH on proliferation of cells derived from rat anterior pituitary gland. The GHRH content of pituitary tissue, cultured pituitary cells, and the conditioned media was evaluated by radioimmunoassay (RIA). Primary cultures of pituitary cells derived from adult rats were prepared by enzymatic dispersion. Significant amounts of GHRH-like molecules were detected in both pituitary tissue and cell cultures by GHRH RIA. Competition curves with increasing amounts of tissue extracts and conditioned media were parallel with those of standard peptide, indicating that the pituitary GHRH-like material is similar to authentic GHRH. To analyze specific cell types responsible for producing GHRH in anteroior pituitary, cell fractionation technique combined with GHRH RIA was performed. In cell fractionation experiment, the highest level of GHRH content was found in gonadotrope enriched-fraction and followed by somatotrope-, lactotrope- and thyrotrope-fraction. Treatment of pituitary cells with GHRH resulted in a dose-dependent increase in [^{3}H] thymidine incorporation. The mitogenic effect of GHRH could be mediated by typical oncogenic activation since the GHRH induced transient increase in *c-fos* mRNA levels with peak response at 30 minutes. The present study demonstrated that i) the pituitary GHRH expressed in the rat anterior pituitary gland can be secreted, ii) among the various cell types, gonadotropes and somatotorpes are the major GHRH source, and iii) the GHRH treatment increased the [^{3}H] thymidine incorporation and *c-fos* transcriptional activity in the pituitary cell culture. These findings suggested that GHRH could participated in the paracrine and/or autocrine regulation of cell proliferation, as well as promoting growth hormone secretion.

Key words: Rat pituitary GHRH, Mitogenic effect.

요 약: 흰쥐 시상하부에서 합성·분비되어 뇌하수체 전엽에서의 growth hormone (GH) 분비를 촉진하는 growth hormone releasing hormone (GHRH)이 시상하부 이외 조직들 (extrahypothalamic tissues)인 태반, 생식소, 그리고 뇌하수체 전엽에서도 발현됨이 보고되었다. 본 연구는 흰쥐 뇌하수체 전엽에서 발현되는 GHRH의 기능을 조사하기 위해 i) 세포 배양을 시행하면서 GHRH의 세포내 함량, 분비 그리고 세포분획법 (cell-fractionation)을 사용하여 분리한 뇌하수체 세포 유형별로 GHRH 함량을 방사면역측정법으로 조사하였고, ii) 체외배양 중인 뇌하수체 전엽세포의 증식에 미치는 GHRH의 효과를 측정하기 위해 [^{3}H] thymidine incorporation assay를, 그리고 iii) GHRH의 세포분열 촉진 효과와 세포내 *c-fos* 유전자 발현과의 상관관계를 조사하기 위해 northern blot analysis를 시행하였다. GHRH 방사면역측정법을 시행한 결과 상당량의 GHRH-like 분자들이 흰쥐 뇌하수체 전엽내에 존재하고, 체외 세포배양시 분비됨을 관찰하였다. 세포분획을 사용한 실험에서 GHRH 함량은 gonadotrope, somatotrope, lactotrope 그리고 thyrotrope 순으로 나타났다. 이러한 결과는 흰쥐 뇌하수체 전엽에서 생성된 GHRH가 국부적인 조절인자, 특히 상이한 유형의 세포들 간의 상호조절 (cross-talk)을 통해 뇌하수체 전엽에서의 세포분열과 분화, 그리고 기능조절에 관여할 가능성을 보여주었다. GHRH는 체외 배양중인 뇌하수체 전엽세포의 [^{3}H] thymidine incorporation을 농도의존적으로 증가시켰으며, 이러한 GHRH의 세포분열 촉진 효과는 예상대로 세포내 oncogene 활성의 증가를 통해 일어나는 것임을 *c-fos* northrn blot으로 확인하였다. 결론적으로, 본 연구는 흰쥐 뇌하수체 전엽에서 합성되는 GHRH가 paracrine 또는 autocrine 기작으로 GH의 분비 촉진 이외에도 세포분열의 조절함을 시사하는 것이다.

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INTRODUCTION

Rat growth hormone releasing hormone (GHRH) is a 43 amino acid hypothalamic peptide that serves as the major regulator of the synthesis and secretion of growth hormone in the anterior pituitary (Frohman and Downs, 1986). Since the first identification of GHRH peptide from the human pancreatic tumor (Guillemin et al., 1982), both GHRH peptide and transcript have been found in several extrahypothalamic sites including testis (Berry and Pescovitz, 1988), placenta (Margioris et al., 1990), ovary (Bagnato et al., 1992) and lymphocytes (Weigent et al., 1991). Recently, the expression of GHRH in rat anterior pituitary gland and pituitary derived tumor cell-lines α T3 and GH3 was confirmed by using RT-PCR, northern blot and immunocytochemistry (Lee, 1998). These new sources and targets for GHRH indicate GHRH may also have some regulatory roles in peripheral sites mostly reproductive tissues. The suggested functions of placental GHRH include role in the regulation of fetal growth hormone and placental lactogen secretion during the embryonic period (Margioris et al., 1990). In the rat gonad, GHRH may act as a positive regulator of gonadotropin-stimulated streroidogenesis and gametogenesis (Moretti et al., 1990; Ciampani et al., 1992; Srivastava et al., 1993). Concerning GHRH, immunoreactive GHRH peptide and GHRH transcript were detected in human pituitary adenomas particulary in the subgroup of somatotrope adenomas (Levy and Lightman, 1992). However, the roles of rat pituitary GHRH have not been explored yet, since the pituitary gland is the classical target tissue of hypothalamic GHRH and discrimination between the authentic and locally produced GHRH is quite difficult.

The aim of the present study was to investigate the possible local role of locally produced GHRH in regulation of rat pituitary function such as cell proliferation and differentiation. Following set of experiments were performed to fulfill the goal; i) to prove that the considerable level of pituitary GHRH can be secreted by cells and to identify the major GHRH producing cell type(s), radioimmunoassay (RIA) was employed using rat anterior pituitary tissue, dispersed cells, conditioned media and fractionated cells, and ii) to test the potent mitogenic effect of GHRH, [^{3}H] thymidine incorporation assay was performed after *in vitro* GHRH treatment.

MATERIALS AND METHODS

1. Preparation and Culture of Rat Anterior Pituitary Cells

Anterior pituitary glands derived from adult female Sprague-Dawley rats (Sangmyung University) were dispersed by trypsin and the cell suspension (3×10^6 cells/ml) was plated in Costar 6-well plates. Dispersed cells were incubated with Dulbeco's Modified Eagle's Medium (DMEM, Sigma) containing 10% fetal bovine serum and antibiotics (GIBCO) for 3 days at 37℃ (Lee, 1998). After preincubation, medium was changed to serum-free DMEM and cells were treated with GHRH peptides (Peninsula, USA) or [^{3}H] thymidine (2.5 μCi/ml; Amersham). For separation of specific anterior pituitary cell types, the dispersed cells were allowed to sediment under unit gravity in grdient chamber (BioRad) containing Percoll solution diluted in medium 199 with 25 mM HEPES and were fractionated into collection tubes (Denef et al., 1989).

2. GHRH RIA

GHRH was measured by RIA as previously described (Lee, 1998). Tissue and cell extracts were homogenized in ice-cold 2M acetic acid, and the homogenates were boiled for 10 minutes and centrifuged at $10,000\times g$ for 10 minutes. The supernatants were neutralized with 2 N NaOH and used for assay. Culture media were similarly acidified and neutralized. GHRH measurements were performed with a specific rat GHRH RIA kit (Peninsula), using about 18,000 cpm of HPLC-purified [^{125}I]-rat GHRH as tracer. The sensitivity of the assay was 4 pg/tube. All samples were assayed in duplicate.

3. [^{3}H] Thymidine Incorporation Assay

After 2 days of preincubation, cells were cultured with [^{3}H] thymidine (2.5 μCi /ml; Amersham) containing Medium 199 supplemented with 1% horse serum for an additional 24 or 48 hours. After the incubation, the radioisotope-contained media were carefully aspirated and the attatched cells were washed twice with ice-cold PBS. The cells were then fixed and disrupted by trypsin and 1% SDS at room temperature for 30 min. The radioactivity of the incorporated [^{3}H] thymidine was measured by scintillation spectrophotometry.

4. *c-fos* and GAPDH Northern Blot Analysis

Total RNAs were extracted from cultured pituitary cells by the acid guanidine thiocyanate-acidic phenol method (Chomzyncski and Sacchi, 1987). The following northrn blot analysis was preformed based on the standard method (Sambrook et al., 1989). Ten ug aliquots of total RNA were electrophoresed in 1.5% denaturing gel and transfered to a Nytran membranes (Schleicher and Schuell). The membranes were hybridized with ^{32}P-dCTP (10 μCi/μl; NEN) labeled *c-fos* or GAPDH cDNA fragments. After several round of washing with SSC and SDS, the intensities of signals on the probe-hybridized filters were determined by a Phosphoimager Analyzer (Clontech) and normalized to GAPDH hybridization signals.

5. Statistical Analysis

Unless otherwise stated, Bar represents the mean ± SE of repeated experiments (n=4~6). The statistical significance of the difference between control and experimental group was assessed by Student's t-test.

RESULTS

The competition curves with increasing amounts of the anterior pituitary extracts were in parallel with that with GHRH standard, indicates the pituitary GHRH-like molecules similar to the authentic GHRH were synthesized in rat pituitary. The content of immunoreactive GHRH in the gland from adult male rat was approximatly 20 pg/tissue (Fig. 1A). Considerable amount of immunoreactive GHRH was detected from both pituitary cell extracts and conditioned-media showing good dose-dependent parallelism with GHRH standard (Fig. 1B). Since the serum-free medium was used for assay after 48 hours of preincubation, the detected GHRH molecules were not originated from hypothalamus or serum in media but were released from the cultured pituitary cells.

When cell fractionation coupled with GHRH RIA was applied to these anterior pituitary cells, highest GHRH content was seen in gonadotrope-enriched fraction followed by somatotrope- and lactotrope-fraction (Fig. 2). This result was in good agreement with the finding that GnRH treatment (1 nM) significantly altered the GHRH secretion from the anterior pituitary cells *in vitro*, implying gonadotorpes are the major GHRH producing cells in the pituitary gland (data not shown).

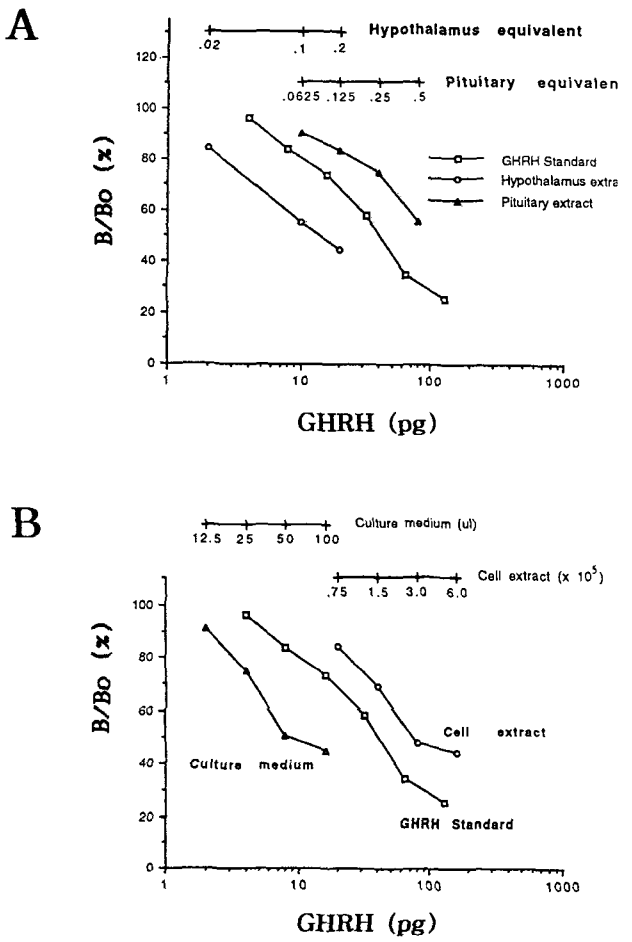


Fig. 1. Presence of immunoreactive GHRH in rat anterior pituitary gland, dispersed cells and conditioned media : GHRH RIA parallelism. A. tissue extracts. B, cell extracts and conditioned media.

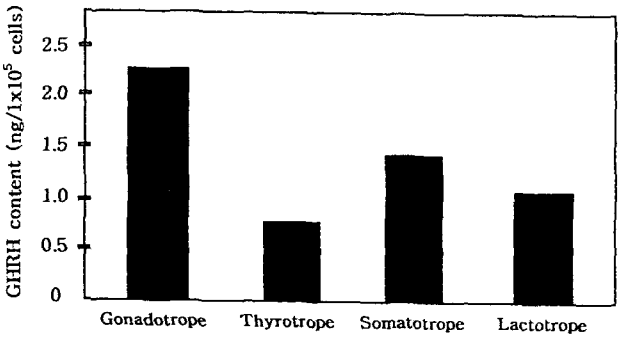
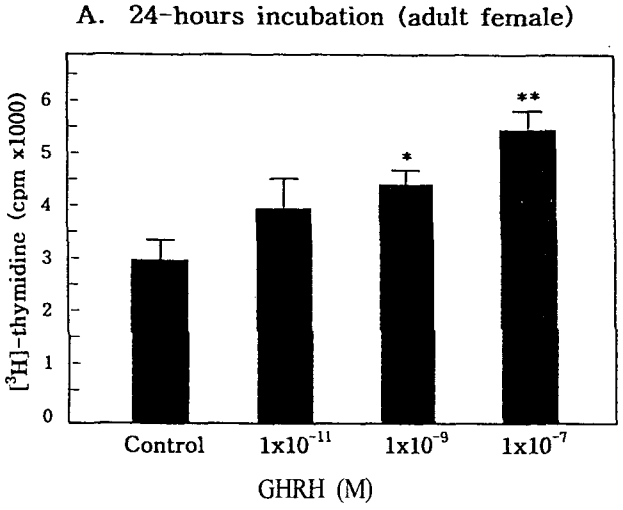


Fig. 2. Identification of the types of anterior pituitary cells that produced GHRH by cell fractionation coupled with GHRH RIA. Bar indicates the mean GHRH value from two independent cell fractionations and RIAs.

Increasing concentration of GHRH (from 10pM~100nM) stimulated the [^{3}H] thymidine incorporation with dose-dependent manner in 24 hours- and 48 hours-cultured anterior pituitary cells from adult female rats (Fig. 3, A and B). The increment of cpm in control group (48 hour vs 24 hours) might be resulted by the action of intrinsic, baseline level GHRH secreted from the cultured cells.

Fig. 4 clearly shown the dramatic induction of *c-fos* expression which was detectable after 15 minutes of stimulation with GHRH (10 nM) in the cultured rat anterior pituitary cells. A maximum response with 9-fold increase was observed after 30



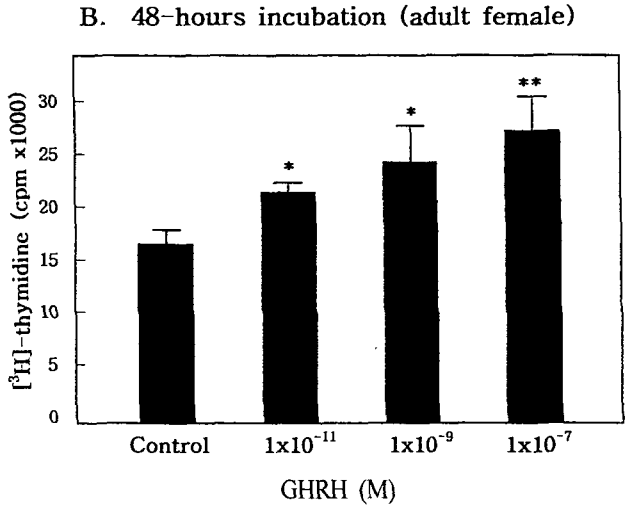


Fig. 3. Mitogenic effect of GHRH on [^{3}H] thymidine incorporation of cultured rat anterior pituitary cells. A. 24 hour incubation. B, 48 hour incubation. *, control vs GHRH-treated groups ($P<0.05$). **, $P<0.01$ (significant difference).

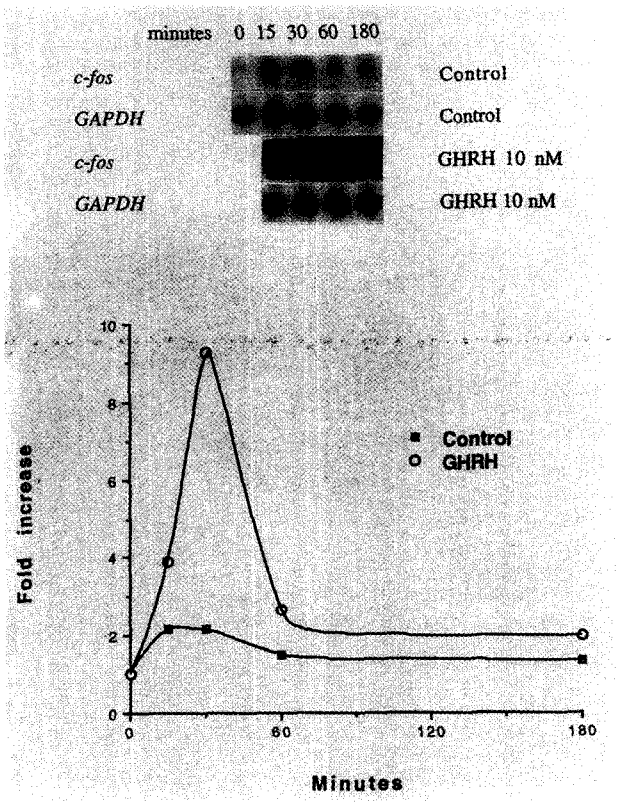


Fig. 4. Stimulatory effects of GHRH on *c-fos* mRNA levels in cultured rat anterior pituitary cells.

minutes of GHRH stimulation, then the transcriptional level sharply declined.

DISCUSSION

An ample of evidence is now available that several hormones or neuropeptides classically associated hypothalamus have been found in the anterior pituitary gland, supporting the existence of local regulatory functions of these hormones (Houben and Denef, 1994). For instance, expression TRH was detected and localized by PCR and in situ hybridization in the rat anterior pituitary gland indicating that there are not only endocrine but paracrine or autocrine regulation of anterior pituitary function by the releasing hormones (Bruhn et al., 1994). Although the precise biochemical nature of these pituitary-derived releasing hormones were not characterized, they may share the specific receptors with authentic hypothalamic peptides and act on fine tuning of anterior pituitary hormone biosynthesis and secretion. Concerning GHRH, immunoreactive GHRH peptide and GHRH

transcript were detected in normal and tumoral pituitary gland from human and rat (Levy and Lightaman, 1992; Lee, 1998). So far, however, evidence for the action of locally produced GHRH in the pituitary gland has not been reported.

The present study could supply the clue for the elucidation of pituitary GHRH function and dynamic nature of pituitary cell differentiation regulated not only by hypothalamic hormones but also by cell-cell interactions in the pituitary gland. Firstly, the GHRH RIA revealed that the amount of immunoreactive GHRH in rat anterior pituitary was approximately 10 times lower than in the hypothalamus, but the levels in pituitary cell extracts and conditioned media might be sufficient for the local action. Since posterior pituitary was eliminated during tissue isolation procedure, the detected immunoreactive GHRH was solely derived from anterior pituitary. The dispersed cells were cultured after 3 days of preincubation in serum-free medium, one could rule out the possibility that the GHRH immunoreactivity was due to the membrane-bound molecules originated from hypothalamus or serum GHRH.

Secondly, the cell-fractionation coupled with GHRH RIA confirmed the previously reproted immunocytochemical study that GHRH staining was found in the cytoplasm of large and medium-sized anterior pituitary cells typically classified as gonadotropes and somatotropes, respectively (Lee, 1998). Interestingly, the highest level of GHRH immunoreactivity was seen in gonadotrope-enriched fraction, followed by somatotrope- and lactotrope-fractions. With the evidence shown the influence of gonadotropin releasing hormone (GnRH) on the PRL and GH secretion *in vitro* (Andries and Denef, 1995) and interaction between alpha T3-1 cells and somatotropes (Andries et al., 1995), the present study suggested the cross-talk between the cells with different type particularly gonadotropes and somatotropes might be important for the understanding of complex pituitary function.

Thirdly, the mitogenic effect of GHRH on the rat anterior pituitary cells was proven in the present study. GHRH treatment increased the rate of proliferation in pituitary cell culture with a dose-dependent manner. Like other hormone and/or growth factors, the mitogenic effect of GHRH might be mediated by activity of early response gene, a cellular oncogene *c-fos*. The *c-fos* transcriptional activity was more effectively elevated by GHRH (10nM) than by GnRH (100 nM) tested in the exactly same culture regimen (Cesnaj et al., 1994). In fact, the transgenic mouse models clearly shown that GHRH overexpression by anterior pituitary cells induced development of pituitary hyperplasia and adenomas particularly GH-oma and prolactinoma (Mayo et al., 1988). In human, the presence of GHRH peptides with different size from authentic hypothalamic form in the normal anterior pituitary and several types of adenoma were demonstrated (Rauch et al., 1995). It is therefore posssible that the abnormal GHRH expression within the anterior pituitary may have an etiological role in the formation of some pituitary adenomas by altering the control of cell proliferation or survival.

In conclusion, the present study demonstrated that the GHRH gene expressed in the rat anterior pituitary gland could serve as a paracrine and/or autocrine regulator of cell proliferation, as well as a stimulus of growth hormone secretion.

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