Endocrine, *vol. 30, no. 1, 55–62, August 2006* 0969–711X/06/30:55–62/$30.00
ENDO (Online) ISSN 1559-0100

Differential Regulation of GHRH-Receptor and GHS-Receptor Expression by Long-Term In Vitro Treatment of Ovine Pituitary Cells with GHRP-2 and GHRH

Sang-Gun Roh,[1] Maria Doconto,[2] Dan Dan Feng,[2] and Chen Chen[2]

[1]Department of Food Production Science, Faculty of Agriculture, Shinshu University, Nagano-ken 5399-4598, Japan; and [2]Endocrine Cell Biology, Prince Henry's Institute of Medical Research, Clayton, Victoria 3168, Australia

GH secretion is regulated by GHRH and somatostatin via actions on their specific receptors in pituitary somatotropes. Ghrelin and synthetic analogs, GHRPs, also stimulate GH release via GHS-receptors (GHS-R). To examine the long-term effect of GHRH and/or GHRP on somatotropes, primary cultured ovine somatotropes were treated with GHRH (10^{-9} and 10^{-8} *M*) and GHRP-2 (10^{-8} and 10^{-7} *M*) for up to 2 d. After treatment, culture medium was collected for GH assay, and total RNA was extracted for RT-PCR analysis. To evaluate cell cultures used in this report, somatotrope-enriched pituitary cells were challenged by 6 h GHRH and dexamethasone (DEX) treatment. As expected, GHRH significantly decreased, whereas DEX increased, the levels of GHRH-R mRNA. Combined low doses of GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) treatment for 24 h increased accumulated GH secretion, significantly more than that induced by high doses of GHRH (10^{-8} *M*) and GHRP-2 (10^{-7} *M*). While levels of GHRH-R mRNA increased, GHS-R mRNA levels were decreased by low doses of GHRH and GHRP-2 for 24 h. High doses of GHRH and/or GHRP-2 for 2 d did not increase GH secretion in the second day of treatment and reduced the level of GHRH-R mRNA. High doses of GHRP-2 treatment decreased the levels of both GHRH-R and GHS-R mRNA. Low doses of GHRH and/or GHRP-2 for 2 d increased the level of GHS-R mRNA without changing GHRH-R mRNA levels. Such treatment also increased ghrelin- (10^{-9} *M*) or ghrelin/GHRH (10^{-9} *M*)–induced GH secretion. These results suggest that low doses of GHRP-2 and GHRH prime somatotropes for stimulation by GHRH and ghrelin.

Key Words: GHRP-2; GHRH; GH; GHRH receptor; GHS receptor.

Received October 21, 2005; Revised April 3, 2006; Accepted April 10, 2006.
Author to whom all correspondence and reprint requests should be addressed: Chen Chen, Endocrine Cell Biology, Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia 15. E-mail: chen.chen@princehenrys.org

Introduction

The synthesis and secretion of growth hormone (GH) from the pituitary gland are mainly regulated by two hypothalamic hormones: GH-releasing hormone (GHRH), which stimulates GH synthesis and secretion, and somatostatin (SRIF), which inhibits GH release. Cloning of the receptor for the synthetic GH secretagogues (GHSs) or GH-releasing peptides (GHRPs) has suggested the presence of an endogenous ligand which may be an important additional regulator of the GH secretion and synthesis system *(1)*. Ghrelin, found initially in rat stomach, has been demonstrated to be an endogenous ligand specific to the GHS-receptor (GHS-R) *(2)*.

Synthetic GHSs are a family of peptidergic and nonpeptidergic compounds that are potent stimulators of GH secretion. Although synthetic GHRPs and endogenous GHRH immediately stimulate GH secretion in vivo and in vitro, different receptors and signal transduction systems are employed in the pituitary somatotropes *(3)*. Intracellular camp mediates GH secretion stimulated by GHRH, whereas the action of GHRPs is likely to be mainly via protein kinase C (PKC) *(4)*. It is reported that GHRPs potentiate GHRH-induced cAMP production through a PKC-independent mechanism in homogeneous populations of cells expressing the cloned GHRH and GHS receptors *(5)*. Although GHS-R is specific for GHRPs, GHRP-2–stimulated GH secretion is totally blocked by combined GHRH-R and GHS-R antisense oligonucleotides treatment, whereas GHS-R antisense oligonucleotide alone evokes only partial reduction in GHRP-2–stimulated GH secretion *(6)*. The importance of the coexistence of GHS-R and GHRH-R on GH secretion is therefore evident. Levels of both GHRH-R and GHS-R in somatotropes would determine the GH secretion in response to either GHRH or GHRPs.

Acute treatment of somatotropes with GHRH and GHRP-2 induces desensitization in their receptors *(6–9)*. It has also been demonstrated that in vitro treatment of cells for 30 min with GHRH and GHRP-2 increased GHRH-R and GHS-R expression, but the increase was short lived and returned to control levels after 2 h treatment *(10)*. However,

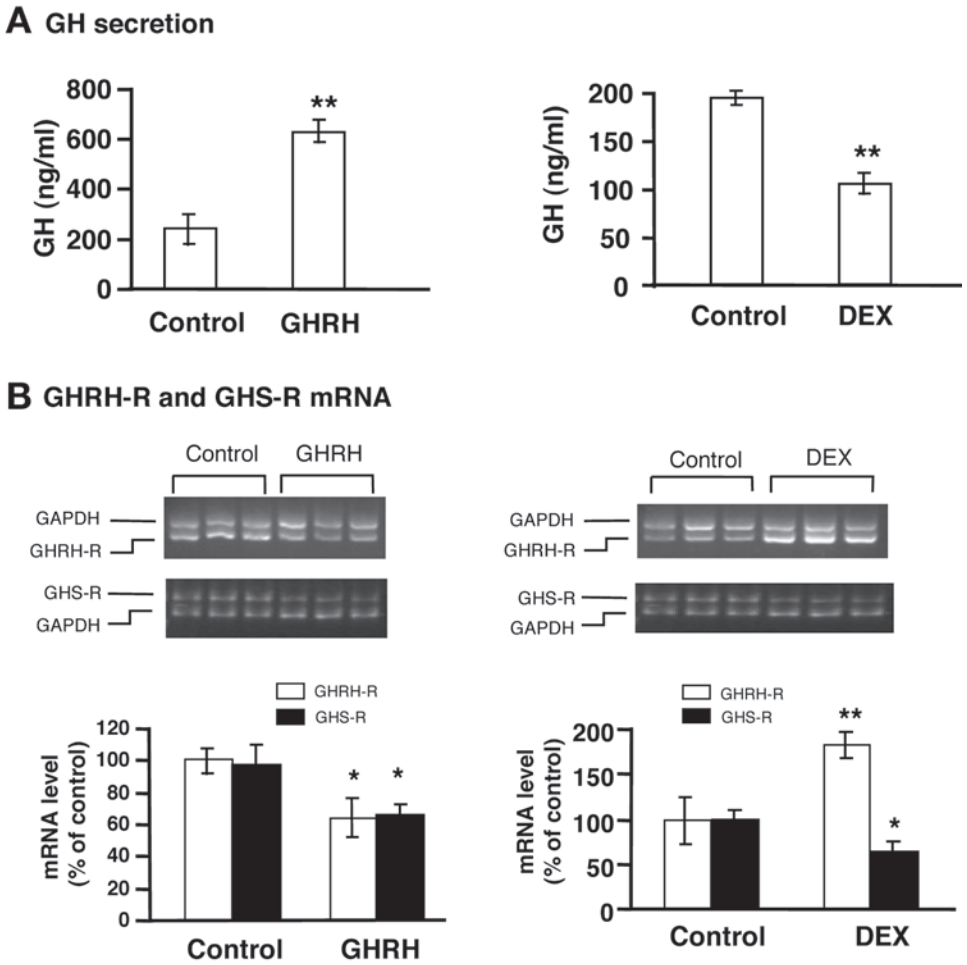

Fig. 1. Effect of GHRH (10^{-8} *M*) and DEX (10^{-7} *M*) treatment for 6 h on GH secretion (**A**), and the levels of GHRH-R and GHS-R (**B**) mRNA in ovine pituitary somatotrope cells. (**A**) Accumulated GH secretion is calculated in incubation medium over 6 h under stimulation of GHRH and DEX. (**B**) Gel panels: upper: EtBr-stained agarose gel displaying amplified GHRH-R (467 bp) and GAPDH (562 bp); lower: EtBr-stained agarose gel showing amplified GHS-R (658 bp) and GAPDH (562 bp). The ratios of GHRH-R or GHS-R mRNA to same sample of GAPDH were calculated to give column figures below the gel panels. GHRH treatment reduced both GHRH-R and GHS-R expression, whereas DEX treatment increased GHRH-R but reduced GHS-R expression. The column represents the mean ± SEM of three separate experiments. $*p < 0.05$, $**p < 0.01$ vs control group.

little is known about the synthesis of GHRH-R and GHS-R under long-term (days) GHRH and GHRP treatment conditions, which are closely related to therapeutic use of GHRP. GHRH also exerts several high peak levels in hypothalamic–pituitary portal circulation each day in vivo to maintain normal condition of somatotropes. This study therefore aims to demonstrate the long-term effect of GHRP-2, with or without GHRH, on ovine somatotropes in vitro.

Results

Effect of GHRH (10^{-8} M) and DEX (10^{-7} M) on Ovine Somatotropes

In order to demonstrate that the cell culture system used in this experiment is a useful experimental model for long-term in vitro treatment, the effect of GHRH and DEX on cell cultures was investigated. It has been well documented that GHRH and DEX regulate GH secretion and GHRH-R synthesis. Our first experiment tested the downregulation of GHRH-R expression and increase in GH secretion by 6 h GHRH treatment (Fig. 1). We then examined the upregulation of GHRH-R by DEX treatment. As expected, based on previous reports, DEX (10^{-7} *M*) decreased GH secretion, but increased the GHRH-R mRNA levels (Fig. 1). We also tested GHS-R mRNA levels in the above experiments, which have not been studied previously. GHS-R mRNA was reduced by both GHRH and DEX treatment (Fig. 1B).

Effects of GHRH (10^{-9}, 10^{-8} M) and GHRP-2 (10^{-8}, 10^{-7} M) Treatment for 24 h on Ovine Somatotropes

As the cell culture system was found to be effective in testing the long-term in vitro treatment by GHRH and DEX, we then tested the effect of 24 h treatment of cells with GHRH and GHRP-2. In our previous experiments, 10^{-8} *M* of GHRH and 10^{-7} *M* of GHRP-2 for up to 60 min induced maximal GH secretion in ovine somatotropes in vitro *(11)*.

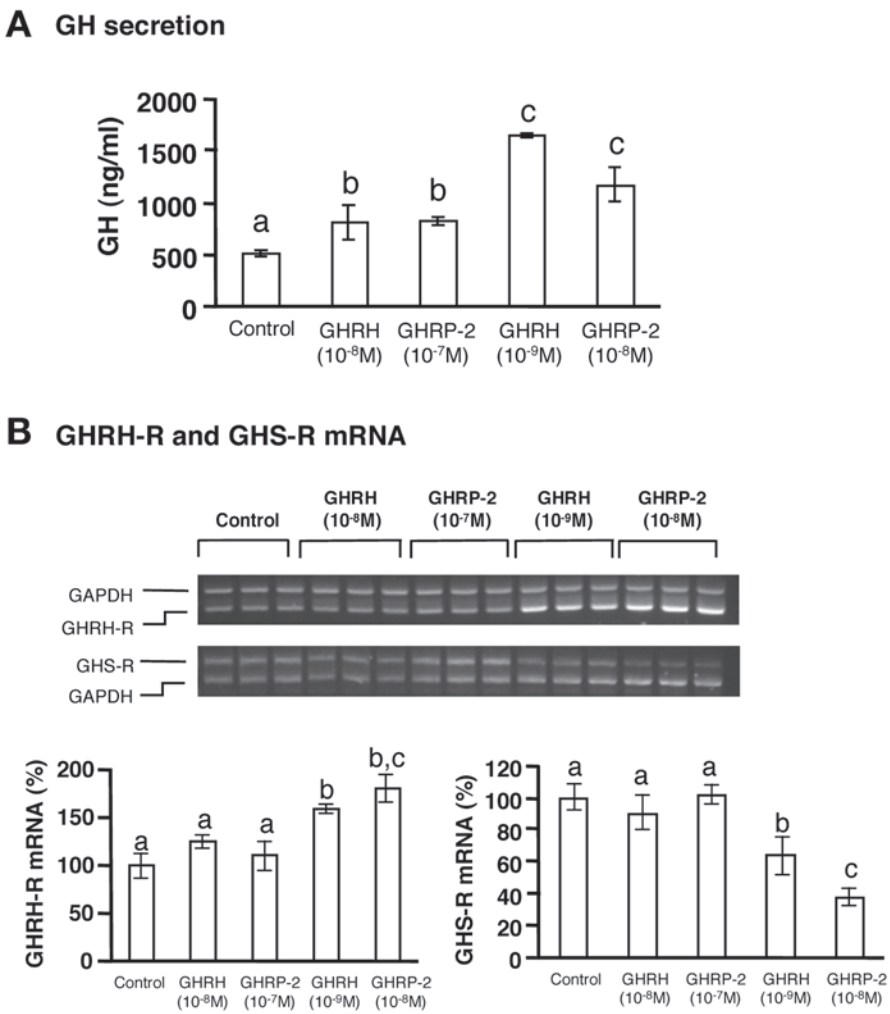

Fig. 2. Effect of GHRH or GHRP-2 treatments for 24 h on GH secretion (**A**), and the levels of GHRH-R and GHS-R mRNA (**B**) in ovine pituitary somatotropes. (**A**) Culture medium was collected for GH assay after cells were incubated with the medium containing different doses of GHRH and GHRP-2. Lower doses of GHRH and GHRP-2 induced more GH secretion in 24 h incubation. (**B**) Gel panels: upper: EtBr-stained agarose gel showing amplified GHRH-R and GAPDH; lower: EtBr-stained agarose gel showing amplified GHS-R and GAPDH. The ratios of GHRH-R or GHS-R mRNA to same sample GAPDH were calculated to give column figures below the gel panels. Lower doses of GHRH and GHRP-2 for 24 h increased GHRH-R mRNA and decreased GHS-R mRNA. The column represents the mean ± SEM of three separate experiments. Statistical significance ($p < 0.05$) between groups are represented by different letters (a, b, or c) above each column. Columns labeled with same letter have no difference.

In this study of 24 h treatment with replenishment every 12 h, 10^{-9} *M* GHRH and 10^{-8} *M* GHRP-2 (close to half-maximal effect doses), induced higher accumulated GH secretion levels than the maximal doses of 10^{-8} *M* GHRH and 10^{-7} *M* GHRP-2 (Fig. 2A). Maximal doses of GHRH and GHRP-2 did not change the level of GHRH-R and GHS-R mRNA, whereas low doses of GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) increased GHRH-R mRNA and decreased GHS-R mRNA (Fig. 2B).

Effects of GHRH (10^{-8} **M***) and/or GHRP-2 (10^{-7}* **M***) Treatment for 48 h on Ovine Somatotropes*

To extend the treatment time, we tested the effect of 48 h treatment with replenishment of GHRH or GHRP-2 every 12 h. Accumulated GH secretion was significantly increased by GHRH and/or GHRP-2 in the first 24 h treatment, but not in the second 24 h (Fig. 3A). GHRH (10^{-8} *M*) treatment reduced GHRH-R mRNA but not GHS-R mRNA levels (Fig. 3B). GHRP-2 (10^{-7} *M*) treatment decreased GHRH-R mRNA and GHS-R mRNA levels. Furthermore, co-treatment of GHRH and GHRP-2 decreased the levels of GHRH-R mRNA with an inhibitory effect similar to GHRH or GHRP-2 alone. This combined treatment kept levels of GHS-R mRNA to control levels, which was significantly higher than that in GHRP-2 alone treatment. It is therefore possible that GHRH maintains GHS-R expression levels in the presence of the agonist of GHS-R, GHRP-2.

Effects of GHRH (10^{-9} **M***) and/or GHRP-2 (10^{-8}* **M***) Treatment for 48 h on Ovine Somatotropes*

In this experiment, we tested the half-maximal doses of GHRH and GHRP-2 treatment on cultured cells. Accumu-

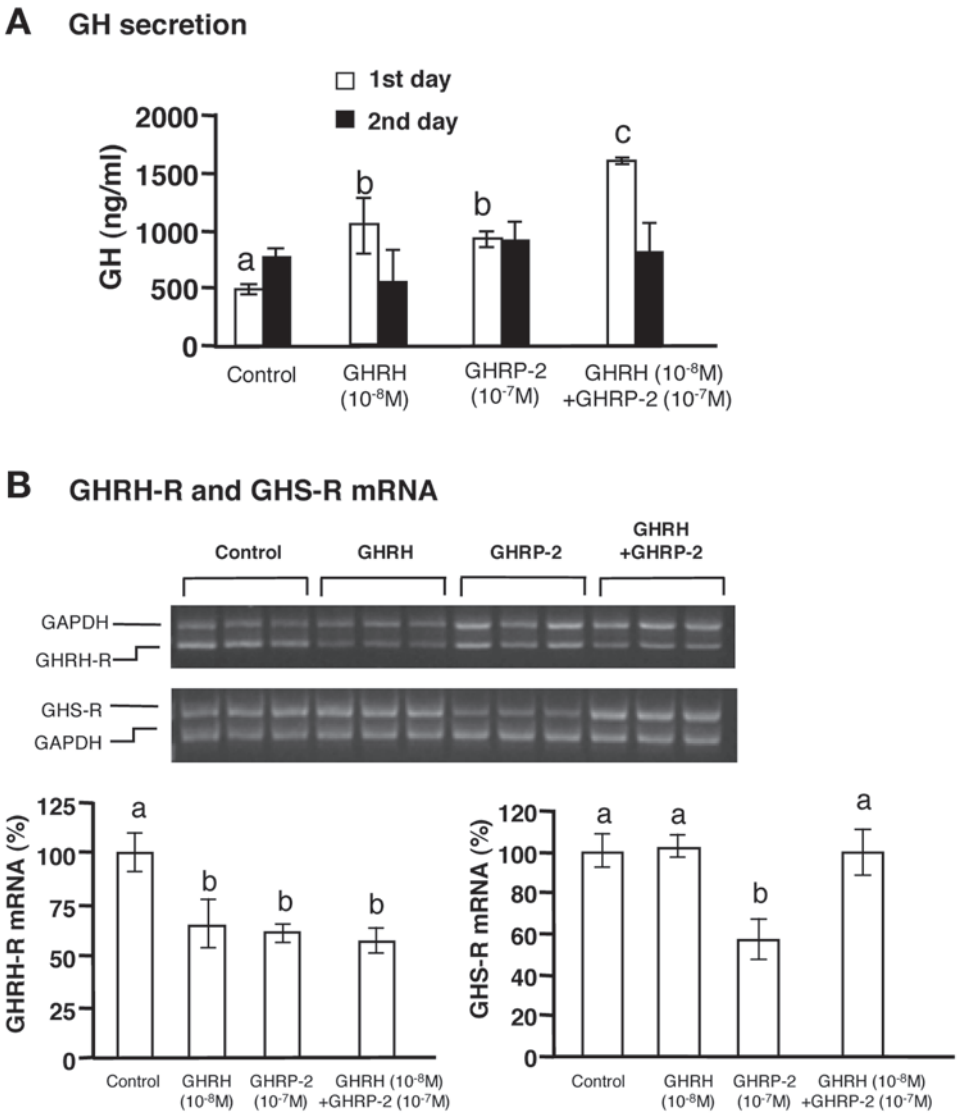

Fig. 3. Effect of high doses of GHRH and/or GHRP-2 treatments for 48 h on GH secretion (**A**), and levels of GHRH-R and GHS-R mRNA (**B**) in ovine pituitary somatotropes. (**A**) Culture medium was collected for GH assay after cells were incubated with the medium containing GHRH and GHRP-2 for first and second 24 h period. First 24 h treatment of cells with GHRH and GHRP-2 induced significant GH secretion whereas no difference was found in second 24 h incubation. (**B**) Gel panels: upper: EtBr-stained agarose gel showing amplified GHRH-R and GAPDH; lower: EtBr-stained agarose gel showing amplified GHS-R and GAPDH. The ratios of GHRH-R or GHS-R mRNA to same sample GAPDH were calculated to give column figures below the gel panels. High doses of GHRH and GHRP-2 for 48 h decreased GHRH-R mRNA and only GHRP-2 treatment decreased GHS-R mRNA. The column represents the mean ± SEM of three separate experiments. Statistical significance ($p < 0.05$) between groups are represented by different letters (a, b, or c) above each column. Columns labeled with same letter have no difference.

lated GH secretion was increased by GHRH and/or GHRP-2 treatment during the first 24 h (Fig. 4A), but was not altered during the second 24 h. Treatment with GHRH or GHRP-2 and combined GHRH and GHRP-2 significantly increased GHS-R mRNA levels (Fig. 4B) without changing the level of GHRH-R mRNA (Fig. 4B).

GH Response to GHRH and/or Ghrelin after 48 h Treatment with Low Doses of GHRH and GHRP-2

As the expression of GHS-R in somatotropes was increased by 48 h GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) treatment, we tested the effect of ghrelin and GHRH on GH secretion following the treatment. After the treatment, GH release induced by 60 min ghrelin (10^{-9} *M*), and ghrelin + GHRH (10^{-9} *M*) was significantly increased (Fig. 5).

Discussion

Short-term (minutes/hours) GHRH and GHRP-2 treatments have been known to result in substantial desensitization of their own receptors in vivo and in vitro *(9,12,13)*. The present study shows that long-term (days) treatment with GHRH and GHRP-2 regulates the synthesis of GHRH-R or GHS-R, although stimulated GH secretion is absent

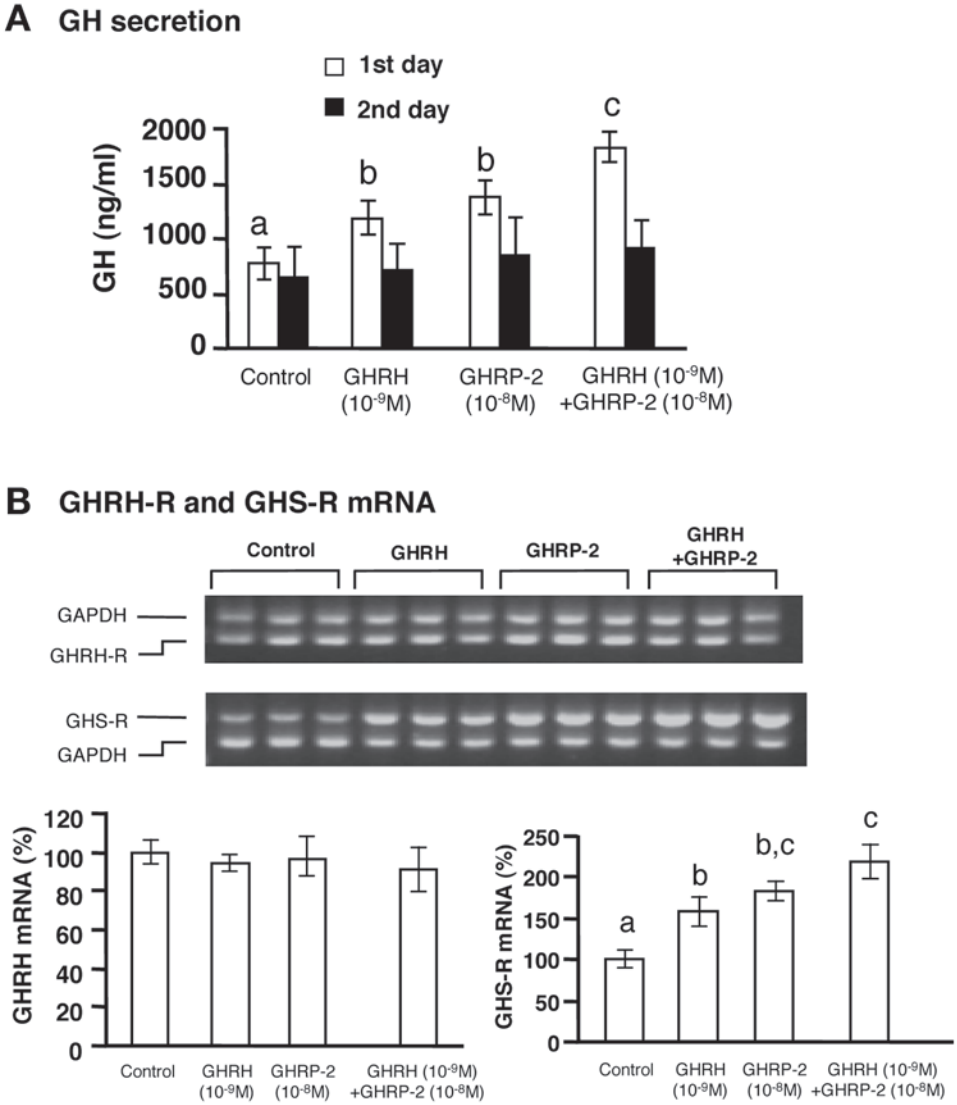

Fig. 4. Effect of low doses of GHRH and/or GHRP-2 treatments for 48 h on GH secretion (**A**), and the levels of GHRH-R and GHS-R (**B**) mRNA in ovine pituitary somatotropes. (**A**) Culture medium was collected for GH assay after cells were incubated with the medium containing GHRH and GHRP-2 for first and second 24 h period. First 24 h treatment of cells with GHRH and GHRP-2 induced significant GH secretion whereas no difference was found in second 24 h incubation. (**B**) Gel panels: upper: EtBr-stained agarose gel showing amplified GHRH-R and GAPDH; lower: EtBr-stained agarose gel showing amplified GHS-R and GAPDH. The ratios of GHRH-R or GHS-R mRNA to same sample GAPDH were calculated to give column figures below the gel panels. Low doses of GHRH and GHRP-2 for 48 h increased GHS-R mRNA without changing GHRH-R mRNA levels. The column represents the mean ± SEM of three separate experiments. Statistical significance ($p < 0.05$) between groups is represented by different letters (a, b, or c) above each column. Columns labeled with same letter have no difference.

after first 24 h treatment of cells, presumably via a rapid desensitization of corresponding receptors. High doses of GHRH (10^{-8} *M*) and GHRP-2 (10^{-7} *M*) negatively regulate the synthesis of corresponding receptors, GHRH-R and GHS-R. Our results with 24 h treatment clearly confirm other reports, in which GHRH-induced desensitization in vitro is associated with a rapid decline in GHRH-R mRNA levels *(14,15)*. Ligand-mediated desensitization can occur via multiple pathways, including the removal of the receptor from the cell surface by internalization, reduction in receptor synthesis by decreasing receptor gene transcription, and/or decreasing receptor mRNA stability *(16)*. Low doses of GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) treatment for 48 h, however, increased expression of GHS-R.

DEX treatment in this experiment confirmed reports in which DEX alone induced GHRH-R gene transcription *(17)*. GHRH-R mRNA is significantly increased by DEX, with a maximal response observed in the presence of 100 n*M* DEX *(18)*. Glucocorticoids stimulate expression of the GHRH-R gene both in vivo and in vitro, providing a likely explanation for the ability of this steroid to enhance pituitary responsiveness to GHRH. Our results did not support claims

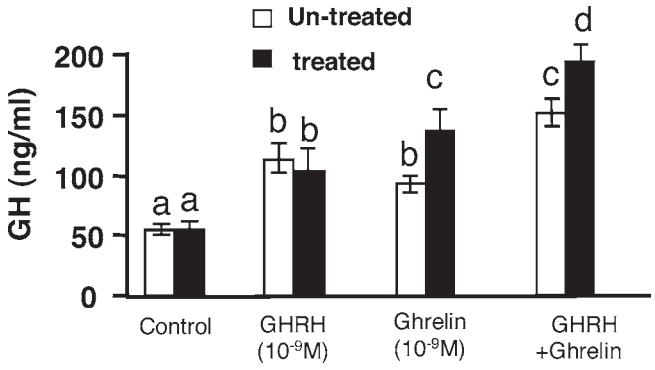

Fig. 5. Effect of 10^{-9} *M* GHRH and/or ghrelin for 60 min on GH secretion from somatotropes with or without 48 h treatment of low doses of GHRH and GHRP-2. After 48 h treatment with GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*), ghrelin and ghrelin+GHRH induced GH secretion, which was significantly higher than that in control group. GHRH-induced GH secretion was not changed. The column represents the mean ± SEM of five separate experiments. Statistical significance ($p < 0.05$) between groups is represented by different letters (a, b, c or d) above each column. Columns labeled with same letter have no difference.

that DEX has a positive effect on GHS-R synthesis in rats *(19)*. This discrepancy between rat and sheep pituitary cells may be due to species differences.

To date, there is limited understanding of the direct and indirect actions of GHRH and GHRP-2 on GHRH-R or GHS-R synthesis, particularly with respect to the effect of long-term treatment. Pituitary GHRH-R and GHS-R expression is normally regulated negatively by GH levels around somatotropes *(20)*. This observation is supported by studies in which the elevated levels of GHRH-R and GHS-R mRNA in the spontaneous dwarf rat were suppressed by GH-replacement therapy *(21,22)*. Furthermore, both GHRH-R and GHS-R mRNA levels observed in the GH receptor/binding protein gene-intact mouse were twice those found in the GH receptor/binding protein gene-disrupted mouse because of the lack of GH negative feedback *(23)*. However, GHRH has been shown to increase GHRH-R mRNA levels in vivo *(24)* where secreted GH is transported away from somatotropes by circulation. GH secretion and response in a static culture system is easily affected by secreted GH from somatotropes. In our culture system, we changed the new culture medium every day to avoid the effects of secreted GH. Furthermore, we added the new GHRH and GHRP-2 after changing the culture medium to avoid their possible degradation over time. The sensitization found under repeated GHRP treatment is not simply due to a depletion of readily releasable pituitary stores of GH, as GHRH can elicit a large GH response following a GHRP injection. This suggests that pulsatile GH secretion from somatotropes was mainly controlled by GHRH, SS, and ghrelin, depending on the level and secretion pattern of these hormones.

It has been clearly demonstrated that GHRP synergizes with GHRH in stimulating GH release in vivo *(25,26)*. However, whether the synergy occurs primarily in the hypothalamus or at the pituitary level is still controversial. Studies using primary cultures of human *(27)*, rat *(28,29)*, and ovine pituitary cells *(8)* showed GHRP was only additive with GHRH to induce GH release from cultured cells. The difference between the in vivo and in vitro effect of GHRP may indicate an indirect action of GHRP on somatotropes existing only under in vivo conditions.

In this study we observed changes in the gene expression of pituitary receptors, which regulate GH synthesis and release. Treatment of cells for 24 h with GHRH and GHRP-2 at low doses, compared with high doses, increased accumulated GH secretion. Negative feedback of GH on somatotropes was observed more than 20 yr ago *(30)*, and this autocrine effect may be responsible for the decrease in GHS-R expression in response to low doses of GHRH and GHRP-2 in this experiment, given that greater accumulated GH secretion occurred. The effect of accumulated GH in the culture medium on GHRH-R and GHS-R is unclear and further experiments with the addition of exogenous GH are required. Interestingly, accumulated GH secretion in the presence of GHRH and GHRP-2 was much less in the second day of treatment, probably due to desensitization of the receptor at that time point. Furthermore, levels of GHRH-R and GHS-R mRNA were decreased by high doses of GHRH and GHRP-2. In contrast, 2-d low doses of GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) increased levels of GHS-R mRNA. These discrepancies can be linked to the receptor desensitization, which is more significant in high doses of agonists *(9,12,13)*. When combined, low doses of GHRH and GHRP-2 treatment significantly increased GHS-R synthesis, showing an additive effect. This suggests that GHRH may activate GHRH-R and signal systems, leading to an increase in the synthesis of GHS-R. This notion is supported by our previous report in which GHRH-R was shown to be not only vital for GHRH-induced GH secretion, but also partially involved in GHRP-2–stimulated GH secretion *(6)*. Furthermore, immuno-neutralization of endogenous GHRH virtually obliterated the GH responses to ghrelin *(31)*. Pandya et al. *(32)* reported that GHRP-6 requires endogenous hypothalamic GHRH for maximal GH stimulation. All these data strongly indicate that the GH response to ghrelin or GHRPs might require an intact GHRH system, and increase in GHS-R expression by GHRH may in part be the reason for this observation.

As GHS-R expression was increased by low does of GHRH and GHRP-2 treatment for 2 d, we tested the GHRH and ghrelin-stimulated GH response to check the function of somatotropes after such treatment. Indeed, ghrelin with or without GHRH induced a significantly higher GH secretion in treated cells. The most logical explanation for this observation is the increase in GHS-R levels in somatotropes. This may lead to, at least partially, a priming effect of GHRP and GHRH on somatotropes to release more GH *(33)*. This result strengthens the potential of GHRPs for therapeutic use.

In summary, the present results indicate that long-term action of GHRH and GHRP may lead to a modification of somatotrope function through regulating expression of GHRH-R and GHS-R. GHRP and GHRH in a low-dose range may prime somatotropes to further stimulation by GHRH and ghrelin.

Materials and Methods

Somatotrope Cell Preparation and Long-Term Treatment with GHRP-2 and/or GHRH

The ovine pituitary glands (from male and female sheep, 3–6 mo of age) were obtained at the time of slaughter from a local abattoir and then subjected to collagenase/pancreatin treatments to liberate cells as described previously. Dispersed ovine pituitary cells were subjected to Percoll gradient centrifugation to enrich the somatotropes (identified by immunocytochemical staining of ovine GH) to up to 80% of total cells *(34)*. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% sheep serum (SS) and 2% fetal calf serum (FCS) in 6-well culture dishes ($1.5–2 \times 10^6$ cells/well). The culture medium was changed every 2–3 d. The treatment of cells with GHRH and dexamethasone (DEX) was performed on the third day of culture. Long-term treatment of cells was performed after 3-4 d in vitro. Culture medium was replaced on the morning of the experimental day by medium containing GHRP-2 (10^{-8} or 10^{-7} *M*) and/or GHRH (10^{-9} or 10^{-8} *M*). The same dose of GHRP and/or GHRH was added at 12-h intervals. Culture medium was replenished daily and collected for GH radioimmunoassay (kept in –20°C); cells were used for RNA extraction. RNA samples were stored in –80°C. For the functional incubation experiment (described below), cells were seeded onto 48-well plates (10^5 cells/well).

GHRH or Ghrelin Challenge after Long-Term Treatment of GHRH and Ghrelin for 2 d

To monitor the GH response to GHRH and ghrelin after long-term treatment with GHRH and GHRP-2, ovine somatotropes were treated with GHRH (10^{-9} *M*) and GHRP-2 (10^{-8} *M*) for 2 d with replenishment every 12 h. After d 2, culture medium in the 48-well culture dishes was changed to incubation medium (without serum, pH value of 7.4 buffered by 15 m*M* HEPES) for the GH secretion assay. Following 1.5 h equilibration at 37°C, incubation medium was discarded and 10^{-9} *M* GHRH and/or ghrelin were included in medium for 60 min incubation at 37°C. The medium was then collected for ovine GH RIA (immediately stored in –20°C).

Semiquantitative RT-PCR for Ovine GHRH-R and GHS-R mRNA

Semiquantitative RT-PCR was performed as previously described *(34)* to measure levels of GHRH-R and GHS-R mRNA. Total RNA from cultured cells was extracted using the single-step acid phenol guanidine protocol *(35)*. RNA samples were treated with Rnase inhibitor and deoxyribonuclease I and quantified by spectrophotometry. The integrity of the RNA samples was assessed by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was reverse transcribed in 20 μL RT reaction system containing random primer and AMV-RT. RT incubations were performed at 46°C. Two microliters from the generated cDNA was used for subsequent PCR amplification for 28–32 cycles, which was in the linear increasing phase of the PCR products. The primers used specific to GHS-R and GHRH-R are (pair 1): GHS-R forward primer (5'-ACCTCCTCTGCAAACTCTTCC-3') and GHS-R reverse primer (5-CACCCGGTACTTCTTGGACAT-3'); (pair 2): GHRH-R forward primer (5'-GCCCGCCTTTCT TCTCTCAC-3') and GHRH-R reverse primer (5'-CTGGG CAATGTGGAGGCTAAG-3'); and (pair 3): GAPDH forward primer (5'-GACCCCTTCATTGACCTCAAC-3') and GAPDH reverse primer (5'-GATGACCTTGCCCACAGC CTT-3'). All PCR for GHRH-R and GHS-R were co-amplified with GAPDH, the housekeeping gene used as an internal control. Twenty microliters of the PCR products was resolved in a 2% agarose gel, the DNA visualized by ethidium bromide (EtBr) staining, and analyzed using Quantity One software (Life Science, New York, NY), where band intensity is expressed in pixels. The ratio levels of ovine GHRH-R and GHS-R mRNA to GAPDH mRNA were calculated and used for figures to reflect the expression levels of GHRH-R and GHS-R. The expression of GPADH mRNA was not altered with any treatments of GHRH and GHRP-2 (data not shown).

Ovine GH Secretion and Radioimmunoassay (RIA)

Before RNA extraction, and after the incubation experiment, culture or incubation medium was collected for ovine GH RIA using materials supplied by the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, USA [oGH (RIA) and oGH antisera]. All samples from one incubation experiment were measured in the same assay. The intraassay and interassay coefficients of variation were less than 10% ($n = 6$) *(34)*. GH values were expressed as ng equivalents of the ovine GH standard.

Statistical Analysis

Values are mean ± SEM of three to five separate experiments with the same treatment protocol. Comparisons were made between different treatment groups using ANOVA followed by Fisher's protected least significant difference as a post-hoc analysis. All experiments conformed to the Ethical code of Practice of National Health and Medical Research Council of Australia.

Acknowledgments

We thank Dr. M. Kojima for supplying ghrelin and Professor C. Bowers for supplying GHRP-2 used in this experi-

ment. We also thank Drs. R. Xu and M. Yan for scientific discussion and Ms. Dianne E. Arnold for editorial help. This work was supported by Australian NHMRC, Aza Australia, and USA Human Growth Hormone Foundation. Dr. Roh was partially supported by Australian Academy of Science and Japanese JSPS under an exchange program.

References

1. Howard, A. D., Feighner, S. D., Cully, D. F., et al. (1996). *Science* **273,** 974–977.
2. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). *Nature* **402,** 656–660.
3. Chen, C., Wu, D., and Clarke, I. J. (1996). *J. Endocrinol.* **148,** 381–386.
4. Cheng, K., Chan, W. W., Barreto, A. Jr., Convey, E. M., and Smith, R. G. (1989). *Endocrinology* **124,** 2791–2798.
5. Cunha, S. R. and Mayo, K. E. (2002). *Endocrinology* **143,** 4570–4582.
6. Roh, S. G., Chen, C., Choi, K. C., Shrestha, Y., and Sasaki, S. (2002). *Endocrinology* **143,** 1964–1967.
7. Hansen, T. K., Moller, J., Thomsen, K., et al. (2001). *Am. J. Physiol. Endocrinol. Metab.* **281,** E1326–E1332.
8. Wu, D., Chen, C., Katoh, K., Zhang, J., and Clarke, I. J. (1994). *J. Endocrinol.* **140,** R9–13.
9. Roh, S. G., He, M. L., Matsunaga, N., Hidaka, S., and Hidari, H. (1997). *J. Anim. Sci.* **75,** 2749–2753.
10. Yan, M., Hernandez, M., Xu, R., and Chen, C. (2004). *Eur. J. Endocrinol.* **150,** 235–242.
11. Wu, D., Chen, C., Zhang, J., Bowers, C. Y., and Clarke, I. J. (1996). *J. Endocrinol.* **148,** 197–205.
12. Clark, R. G., Carlsson, L. M., Rafferty, B., and Robinson, I. C. (1988). *J. Endocrinol.* **119,** 397–404.
13. DeBell, W. K., Pezzoli, S. S., and Thorner, M. O. (1991). *J. Clin. Endocrinol. Metab.* **72,** 1312–1316.
14. Kineman, R. D., Kamegai, J., and Frohman, L. A. (1999). *Endocrinology* **140,** 3581–3586.
15. Aleppo, G., Moskal, S. F., De Grandis, P. A., Kineman, R. D., and Frohman, L. A. (1997). *Endocrinology* **138,** 1058–1065.
16. Grady, E. F., Bohm, S. K., and Bunnett, N. W. (1997). *Am. J. Physiol.* **273,** G586–G601.
17. Nogami, H., Hiraoka, Y., Matsubara, M., et al. (2002). *Endocrinology* **143,** 1318–1326.
18. Miller, T. L. and Mayo, K. E. (1997). *Endocrinology* **138,** 2458–2465.
19. Tamura, H., Kamegai, J., Sugihara, H., Kineman, R. D., Frohman, L. A., and Wakabayashi, I. (2000). *J. Neuroendocrinol.* **12,** 481–485.
20. Nass, R., Gilrain, J., Anderson, S., et al. (2000). *Endocrinology* **141,** 2084–2089.
21. Kamegai, J., Unterman, T. G., Frohman, L. A., and Kineman, R. D. (1998). *Endocrinology* **139,** 3554–3560.
22. Kamegai, J., Wakabayashi, I., Miyamoto, K., Unterman, T. G., Kineman, R. D., and Frohman, L. A. (1998). *Neuroendocrinology* **68,** 312–318.
23. Peng, X. D., Park, S., Gadelha, M. R., et al. (2001). *Endocrinology* **142,** 1117–1123.
24. Girard, N., Boulanger, L., Denis, S., and Gaudreau, P. (1999). *Endocrinology* **140,** 2836–2842.
25. Bowers, C. Y., Reynolds, G. A., Durham, D., Barrera, C. M., Pezzoli, S. S., and Thorner, M. O. (1990). *J. Clin. Endocrinol. Metab.* **70,** 975–982.
26. Penalva, A., Carballo, A., Pombo, M., Casanueva, F. F., and Dieguez, C. (1993). *J. Clin. Endocrinol. Metab.* **76,** 168–171.
27. Shimon, I., Yan, X., and Melmed, S. (1998). *J. Clin. Endocrinol. Metab.* **83,** 174–178.
28. Sartor, O., Bowers, C. Y., and Chang, D. (1985). *Endocrinology* **116,** 952–957.
29. Blake, A. D. and Smith, R. G. (1991). *J. Endocrinol.* **129,** 11–19.
30. Advis, J. P., White, S. S., and Ojeda, S. R. (1981). *Endocrinology* **108,** 1343–1352.
31. Tannenbaum, G. S., Epelbaum, J., and Bowers, C. Y. (2003). *Endocrinology* **144,** 967–974.
32. Pandya, N., DeMott-Friberg, R., Bowers, C. Y., Barkan, A. L., and Jaffe, C. A. (1998). *J. Clin. Endocrinol. Metab.* **83,** 1186–1189.
33. Cella, S. G., Cerri, C. G., Daniel, S., et al. (1996). *J. Gerontol. A Biol. Sci. Med. Sci.* **51,** B439–B447.
34. Roh, S. G., Nie, G. Y., Loneragan, K., Gertler, A., and Chen, C. (2001). *Endocrinology* **142,** 5167–5171.
35. Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem.* **162,** 156–159.