

EXPERIMENTAL STUDY

Effect of GHRH and GHRP-2 treatment *in vitro* on GH secretion and levels of GH, pituitary transcription factor-1, GHRH-receptor, GH-secretagogue-receptor and somatostatin receptor mRNAs in ovine pituitary cells

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Abstract

Objective: Growth hormone (GH)-releasing hormone (GHRH) and GH-releasing peptides (GHRPs) stimulate the release of GH through their specific receptors on somatotropes. Combined GHRH and GHRP administration causes a synergistic GH release *in vivo* by an unknown mechanism. The current study focuses on the direct action of GHRH and GHRP on several molecular targets in somatotropes. **Design and methods:** To clarify the mechanism of action, ovine somatotropes were used to measure the expression of mRNAs encoding for GH, pituitary transcription factor-1 (Pit-1), GH-secretagogue receptor (GHS-R), GHRH-R, somatostatin receptor subtypes (sst-1 and sst-2) and GH release after GHRH and GHRP-2 treatment for 0.5, 1, 1.5 and 2 h.

Results: GHRH (10 nM), GHRP-2 (100 nM) and combined GHRH–GHRP-2 increased the levels of GH mRNA and GH release from 0.5 to 2 h in a time-dependent manner. The levels of Pit-1, GHRH-R and GHS-R mRNA were increased after 0.5 h treatment of cells with GHRH and GHRP-2. The levels of sst-1 but not sst-2 mRNA were significantly increased after 0.5 and 1 h of GHRH treatment. In contrast, both sst-1 and sst-2 mRNA expression was inhibited after 0.5–2 h of GHRP treatment.

Conclusions: These data demonstrate a direct *in vitro* modification of ovine somatotropes by GHRH and GHRP-2 resulting in altered GHRH-R, GHS-R, Pit-1, sst-1, sst-2 and GH gene expression; this may underlie the regulatory action of GHRH and GHRP-2 on GH secretion.

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Introduction

Pituitary growth hormone (GH) expression and secretion are mediated by two hypothalamic peptides, GH-releasing hormone (GHRH) and somatostatin (somatotropin release inhibiting factor, SRIF) and by a peptide of stomach origin, ghrelin (1, 2). Ghrelin acts on the GH secretagogue receptor (GHS-R), which was identified in pituitary gland using synthetic GHSs, including GH-releasing peptides (GHRPs) and non-peptide compounds (MK0677 and L-692585), as ligands (3). GHS administered alone or in combination with GHRH stimulate GH secretion in a number of species including human (4–6). The GHS therefore has the potential to be developed as an effective drug for enhancing circulating levels of GH in patients with intact hypothalamo-pituitary function (7, 8). In clinical diagnosis, combined GHRP and GHRH administration may be considered as the new 'gold standard' test of GH deficiency, with or without hypothalamo-pituitary dysfunction (9). As GHSs directly stimulate GHS-R in the pituitary and hypothalamus to release GH, they do

not bypass the feedback loop controlling the GH–IGF-I (insulin-like growth factor I) axis. Administration of GHS is therefore clinically preferable to the direct administration of GH (10). Furthermore, acting as functional SRIF antagonists, GHSs potentiate the actions of GHRH on GH secretion, enhancing pulsatile GH secretion (11). GHSs therefore could provide a well-tolerated clinical approach for treating or preventing GH deficiency, in conditions such as obesity and in the aging population. Such therapeutic efficacy would have great social impact (11). In addition, as the mechanism of GHS action on the modification of pituitary GH-secreting cells (somatotropes) is at present unclear, a greater understanding of this action may lead to the development of improved GHSs.

GHRH, GHS and SRIF receptors stimulated by GHRH, GHS and SRIF in pituitary somatotropes play critical roles in mediating GH synthesis and release (10). Regulation of the expression of the GH gene is usually mediated by pituitary transcription factor-1 (Pit-1) (12). Pit-1 expression in monolayer cultures of infant rat anterior pituitary cells is increased by GHRP-6

(an early version of synthetic GHS) mediated via the GHS-R (13). GHRH stimulates Pit-1 expression in rat pituitary cells, presumably through action on GHRH-R (14). The expression of GHRH-R has also been linked to the expression of Pit-1 (15, 16), and impaired Pit-1 binding can reduce expression of the GHRH-R gene (17). For the inhibitory hormone SRIF, GHRH treatment increases the expression of pituitary SRIF receptor subtypes (sst-1 and sst-2) (18). However, available data on the effect of GHRH/GHRH-R and GHRP/GHS-R are controversial. GHRH treatment down- or upregulated expression of its receptor by rat pituitary cells cultured in serum-free or serum-containing medium respectively (19, 20). GHS reduced or did not alter GHS-R mRNA expression in pituitary cells in serum-free medium (19). These differences may depend on the duration of treatment of cells with GHRH and GHS, cell culture conditions or the age of the animals used. These factors are critical in assessing the effect of GHRH and GHS on somatotropes and GH secretion, however they have not been systematically studied (20). Pituitary GH is secreted in a highly episodic pulsatile pattern in all species in which it has been examined. This pattern is tightly controlled by the interplay of GHRH, SRIF and probably ghrelin as well. The contribution and physiological change of the corresponding pituitary receptors and GH remain to be elucidated (21).

The current investigation focuses on the regulation of GHRH-R, GHS-R, Pit-1, sst-1, sst-2 and GH gene expression, and GH secretion, by GHRH- and GHRP-2 (the latest GHS construct)-treated ovine pituitary cells, with the aim of clarifying the modification of somatotropes by GHRH and GHRP which leads to a stimulation of GH secretion.

Materials and methods

Ovine pituitary cell cultures

The 18 ovine pituitary glands (from 3–6-month-old sheep) were obtained at the time of slaughter from a local abattoir and then subjected to collagenase/pancreatin treatments to liberate cells as described previously (22, 23). The cell suspension was plated onto 35-mm Petri dishes (2×10^6 cells/well) and allowed to grow as a monolayer. Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% sheep serum, 2% fetal calf serum, penicillin/streptomycin and 1% (v/v) L-glutamine (200 mM). Cultures were maintained in a humidified incubator (37°C, air 95%; CO₂ 5%).

GHRH and GHRP-2 treatment

Treatment of cells with GHRH, GHRP or combined GHRH and GHRP was performed after 24 h culture. On the day of the experiment the culture medium

was changed to serum-free incubation medium. Serum-free DMEM was supplemented by insulin, transferrin, selenium, BSA and linoleic acid. According to the GH secretion and mRNA expression of target genes in response to GHRH and GHRP-2 treatment in cultured pituitary cells from published papers (20, 24, 25), the final concentrations chosen for the incubation medium were 10 nM GHRH and 100 nM GHRP-2. GHRH, GHRP-2 or both were added to replenished incubation medium for 0.5, 1, 1.5 and 2 h. The control group cells received vehicle alone. For all experiments, one incubation with all time points was performed on the same batch of cells to extract total RNA and culture medium to test GH secretion within each experimental treatment (GHRH, GHRP-2 or combined).

Radioimmunoassay for GH

The concentration of GH in the cell incubation medium was measured in duplicate by a double-antibody radioimmunoassay using kits provided by the US National Hormone and Pituitary Program, National Institutes of Health (ovine GH and ovine GH antisera). The ovine GH peptide NIDDK-oGH-I-5 for iodination purposes and standard preparation was supplied by the US National Hormone and Pituitary Program. A set of standards ranging from 0.5 to 50 ng/ml were prepared and included in every assay to determine relative GH amounts. The samples were measured against those values and a set of quality controls included to evaluate any differences between assays. The sensitivity of the assay was 0.3 ng/ml and the inter- and intra-assay coefficients of variation were less than 15 and 8% respectively ($n = 6$). All samples from one experiment were measured in the same assay, and GH values were expressed as nanogram equivalents of the ovine GH standards supplied by the US National Hormone and Pituitary Program.

Semi-quantitative reverse transcription (RT)-PCR analysis

Extraction of total RNA from cells was performed on six-well (35 mm diameter well) culture dishes (2×10^6 cells/well) for RT-PCR reaction using a method described previously (23, 26). After precipitation and washing with ethanol, the total RNA extracted from each culture well was treated with deoxyribonuclease-I (Roche) to eliminate possible contamination by genomic DNA. RT was performed using reverse transcription kits (Promega). Briefly, 1 µg total RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase using random hexanucleotide primers in a 20 µl reaction volume. The RT reactions were carried out at 46°C for 2 h; 1 µl of the RT reaction product was used for subsequent PCR amplification, using specific sets of primers (Table 1) in master PCR buffer (Promega). In preliminary

Table 1 Sequences of primers used for PCR of sheep pituitary GH, Pit-1, GHRH-R, GHS-R, sst-1, sst-2 and β -actin mRNA.

Target gene (GeneBank no.)	Sequences*	PCR product (bp)
GH (S50877)		
Forward	5'-CCTGCTCCTGGCTTTCACCCT-3' (27–47)	439
Reverse	5'-ATCTTCCAGCTCCCGCATCAG-3' (465–445)	
Pit-1 (OAU88399)		
Forward	5'-GGAAACCAGTCATCGACCTATG-3' (181–202)	276
Reverse	5'-ATTTGTCTGGGTGTATCCTAGC-3' (456–435)	
GHRH-R (AY008834)		
Forward	5'-GCCCGGCTTTCTTCTCTCAC-3' (383–402)	477
Reverse	5'-GTGGGCAATGTGGAGGCTAAAG-3' (859–841)	
GHS-R (AY093950)		
Forward	5'-CAG AGA CCA GAA CCA CAA-3' (824–841)	176
Reverse	5'-AGA GGA CGA AAG AGA CGA G (999–981)	
sst-1 (AJ314853)		
Forward	5'-TCTTCCGCAATGGCACCTG-3' (998–1016)	278
Reverse	5'-CAAGGGAGGAAGAAGCGGAAG-3' (1275–1255)	
sst-2 (AF335550)		
Forward	5'-TCTGAGAAGAAGTCCACACG-3' (10–29)	185
Reverse	5'-AGGATAGGGTTGGCACAG-3' (194–177)	
β -Actin (OAU39357)		
Forward	5'-TGGGACGACATGGAGAAGATCTGGC-3' (321–345)	186
Reverse	5'-CAGCACAGCCTGGATGGCCACGTAC-3' (506–482)	

* Sequences of primers used for PCR of sheep pituitary GH, GHRH-R were previously reported (27), and sequences of primers for Pit-1, GHS-R, sst-1, sst-2 and β -actin were designed by the software of NetPrimer (PREMIER Biosoft International, CA, USA).

experiments, the linear phase of the PCR reaction was determined for each set of primers and appropriate cycle numbers were chosen to measure the expression of target genes. All transcripts were amplified with similar efficiencies: between 20 and 30 cycles for GH, Pit-1 and β -actin; between 25 and 35 cycles for GHRH-R, sst-1 and sst-2; between 30 and 40 cycles for GHS-R (data not shown). Similar results were obtained in at least three independent experiments of at least four wells. The validity of the above RT-PCR assays for semi-quantitative analysis is supported by: (i) the selection, for each target, of amplification conditions in the exponential phase of PCR products; (ii) amplification efficiencies being similar between all transcripts within each reaction; and (iii) the use of an appropriate internal control. Thus, PCR amplification was conducted using: 25 amplification cycles for GH, Pit-1 and β -actin transcripts; 30 cycles for GHRH-R, sst-1, and sst-2 transcripts; and 35 cycles for GHS-R. The thermal cycling profile was as follows: 94 °C for 5 min, 25 (GH, Pit-1, β -actin) or 30 (GHRH-R, sst-1, sst-2) cycles of 94 °C for 30 s, 62 °C (GH) or 58 °C (Pit-1, GHRH-R, sst-1) or 54 °C (β -actin, sst-2) for 30 s, 72 °C for 45 s and 72 °C for 10 min. The thermal cycling profile for GHS-R was 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 min and 72 °C for 10 min. The PCR products were separated on a 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Non-reverse transcribed RNA was included with the primer sets as a negative control (28). Amplification of a portion of the β -actin (house keeping) gene served as a control for the efficiency of RNA isolation and cDNA synthesis. GH, Pit-1, GHRH-R, GHS-R, sst-1 and sst-2 mRNA levels

were normalized to the corresponding β -actin mRNA level, as previously described (23).

Data analysis

Data are presented as a percentage of the control, which was set at 100%, and are expressed as means \pm S.E.M. Differences in the effects of GHRH, GHRP-2 or GHRH and GHRP-2 combined on GH concentration and GH, Pit-1, GHRH-R, GHS-R, sst-1 and sst-2 mRNA levels were determined by ANOVA, and percentage changes were analysed by the Kruskal–Wallis H test for difference from controls. *P* values less than 0.05 were considered significant.

Results

Effects of GHRH, GHRP-2, or both, on GH secretion and GH mRNA expression

Accumulated GH release in the incubation medium, in response to GHRH (10 nM) treatment significantly enhanced GH release by up to 303, 296, 319 and 406% of control levels at 0.5, 1.0, 1.5 and 2.0 h after treatment respectively. GHRP-2 (100 nM) significantly stimulated GH secretion up to 388, 234, 369 and 373% of control levels at 0.5, 1.0, 1.5 and 2.0 h after treatment respectively (Fig. 1). The combination of 10 nM GHRH and 100 nM GHRP treatments significantly increased GH release up to 404% 0.5 h after treatment commenced. Thereafter GH release decreased but still remained higher than control levels (203% of control) at 1 h, before ascending up to 353% at 1.5 h and 412% of control 2 h after treatment (Fig. 1).

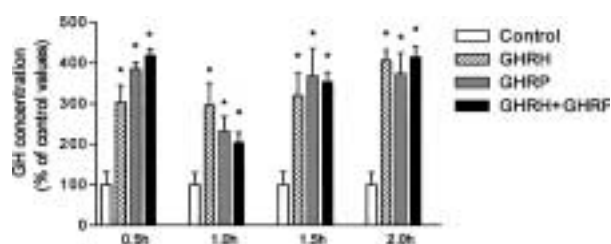


Figure 1 Relative levels of GH concentration in culture medium of ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; $*P < 0.05$ compared with control levels of GH concentration.

GHRH (10 nM) and GHRP-2 (100 nM) treatment of ovine pituitary cells significantly increased the levels of GH mRNAs 0.5, 1, 1.5 and 2 h after treatment (Fig. 2). The combination of 10 nM GHRH and 100 nM GHRP-2 significantly increased the levels of GH mRNA in cells over 0.5–2 h in a time-dependent manner (Fig. 2).

Effects of GHRH, GHRP-2, or both, on Pit-1, GHRH-R and GHS-R mRNA expression

Pit-1 mRNA expression was progressively enhanced to: 139 and 133% at 1 and 2 h respectively after GHRH treatment; 193 and 171% at 0.5 and 2 h respectively after GHRP treatment; and 150% 0.5 h after combined GHRH and GHRP treatment (Fig. 3). The levels of GHRH-R mRNA were significantly increased to 131% 0.5 h after GHRH treatment, but were not significantly affected after GHRP treatment at any time point (Fig. 4). The combined treatment enhanced the levels of GHRH-R mRNA at 0.5, 1.5 and 2 h after treatment (Fig. 4). GHRH (10 nM) treatment did not affect the levels of GHS-R mRNA in ovine pituitary cells (Fig. 5). However, GHRP-2 treatment upregulated mRNA expression of its own receptor by 151% of control 0.5 h after treatment, thereafter declining to control levels at 1 and 1.5 h time points, before returning to 120% of control 2 h after treatment (Fig. 5). The combined

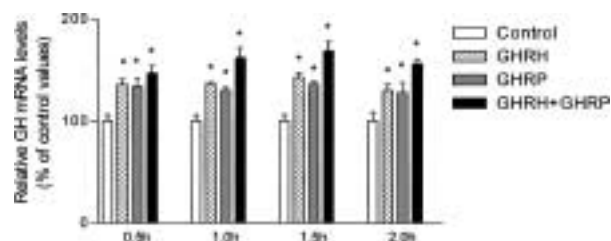


Figure 2 Relative levels of GH mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; $*P < 0.05$ compared with control levels of GH mRNA.

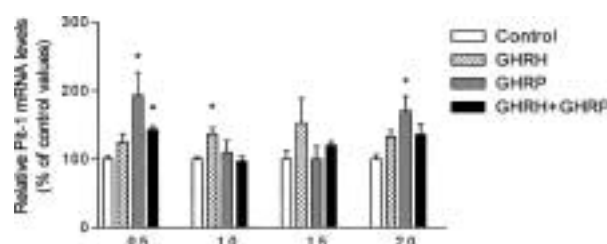


Figure 3 Relative levels of Pit-1 mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; $*P < 0.05$ compared with the control levels of Pit-1 mRNA.

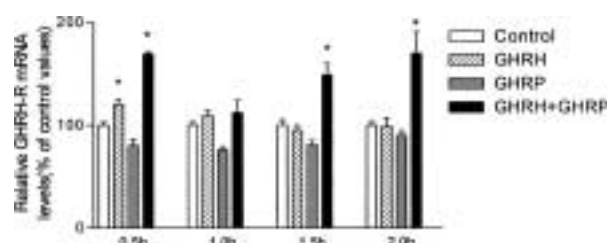


Figure 4 Relative levels of GHRH-R mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; $*P < 0.05$ compared with control levels of GHRH-R mRNA.

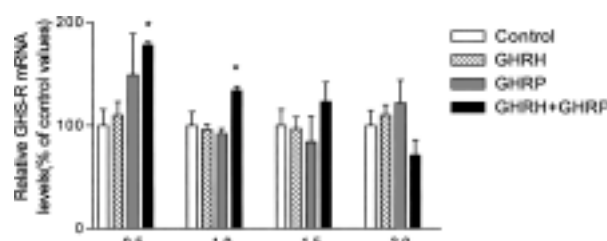


Figure 5 Relative levels of GHS-R mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; $*P < 0.05$ compared with the control levels of GHS-R mRNA.

treatment significantly enhanced the levels of GHS-R mRNA at 0.5 and 1 h, the increase reducing to control levels for GHS-R mRNA at 2 h (Fig. 5).

Effects of GHRH, GHRP-2, or both, on sst-1 and sst-2 mRNA expression

GHRH (10 nM) treatment significantly increased the levels of sst-1 mRNA at 0.5 (180%) and 1 h (193%) after treatment; thereafter levels declined to 140% at 1.5 h and 127% at 2 h after treatment (Fig. 6).

Although 10 nM GHRH increased sst-2 expression at 1 (131%), 1.5 (158%) and 2 h (123%) after treatment, these increases were not statistically significant (Fig. 7). GHRP-2 (100 nM) decreased the mRNA levels of sst-1 and sst-2, but the differences did not reach statistical significance. Likewise the combined GHRH and GHRP treatments did not significantly affect sst-1 and sst-2 mRNA expression (Figs 6 and 7).

Discussion

The most significant findings of this study are that treatment of ovine pituitary cells *in vitro* with GHRH and GHRP regulates GHRH-R, GHS-R, Pit-1, sst-1, sst-2 and GH gene expression, and GH secretion, in a time-dependent manner. GH secretion was significantly increased by combined GHRH and GHRP treatment, suggesting that these joint stimuli afford near-maximal stimulation of somatotropes and may form the basis of tests or treatments for GH deficiency in obesity, children and aging populations (29–32). Therefore, an understanding of the direct effects of GHRH/GHRP on the pituitary and their molecular targets is very important.

Pituitary GH secretion is, to a large extent, controlled by three regulatory hormones: GHRH, GHS and SRIF. Each binds to G-protein-coupled membrane receptors through which intracellular signalling systems are

activated (1). GHRH and GHRP administration potently increases GH secretion and this is not altered by gender, adiposity or age (9). However, peripheral circulating GHRH levels are not usually linked to an increase in GH levels as evidenced in patients with hypothalamic GHRH-secreting tumors (33). The key cell type in the regulation of GH levels is the pituitary somatotrope, which determines the amount of GH secreted in response to hypothalamic GHRH stimulation. Combined GHRH and GHRP treatment plays an important role clinically, however, the data on the mechanism of GHRH/GHRH-R and GHRP/GHS-R action are controversial (19–21). The differences may relate to the duration of GHRH and GHRP treatment and cell culture conditions as well as animal age. In this study, we investigated the mechanism of action of GHRH/GHRP using primary cultures of ovine pituitary cells treated with GHRH and GHRP-2 *in vitro*. The present study carefully investigated the concentration and duration of GHRH and GHRP treatment using 0.5, 1, 1.5 and 2 h time points and serum-free incubation conditions for cell culture to clarify the mechanism of GHRH/GHRH-R and GHRP/GHS-R action. Treatments with 10 nM GHRH and 100 nM GHRP-2 for 0.5, 1, 1.5 and 2 h were chosen in this study to investigate short- to mid-term changes in somatotropes. GHRH at 1.0 µg/kg (i.v.) *in vivo* maximally stimulated GH secretion at 15–45 min, with GH levels returning to baseline by 90–120 min after GHRH injection in humans (34). GHRP-2, GHRP-6, hexarelin or non-peptidyl GHRP mimetic compound (L-692, 429) treatment rapidly increases serum GH concentrations within 5–15 min, with the peak GH concentration usually observed 15–30 min after intravenous injection in humans (35, 36). The presence of serum in the culture medium maintains basal levels of the GHRH receptor and is important in long-term GHRH treatment (20). However, the biological half-life of GHRH 1–44 is about 3–6 min *in vivo* (37) as GHRH is rapidly inactivated by a plasma dipeptidyl aminopeptidase, producing a more stable metabolite, GHRH 3–44, which is about 1000 times less potent than the parent compound (37). The culture of ovine pituitary cells in serum-free culture conditions, as employed in this study, overcomes the quick degradation of GHRH and allows subsequent stimulatory GHRH and GHRP treatments to be performed under defined conditions (38).

GHRH and GHRP treatment for 15 and 30 min rapidly stimulates maximal GH release (34–36). Furthermore, GHRH treatment for as little as 10 min rapidly increased GH transcription rate by 200–300% in primary cultured pituitary cells (39, 40). To analyse the transcription regulation of GH, we examined GH mRNA levels in response to GHRH and GHRP-2. Our results show that treatment with GHRH, GHRP-2 and combinations of GHRH and GHRP-2 increased GH mRNA expression and GH release 0.5, 1.0, 1.5 and 2 h after treatment, in a time-dependent manner.

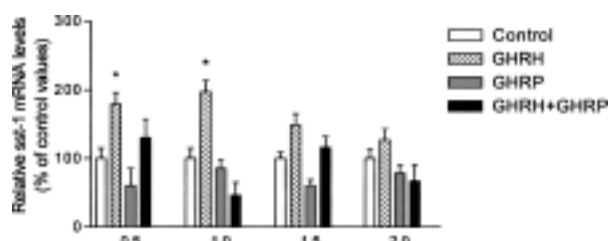


Figure 6 Relative levels of sst-1 mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; * $P < 0.05$ compared with control levels of sst-1 mRNA.

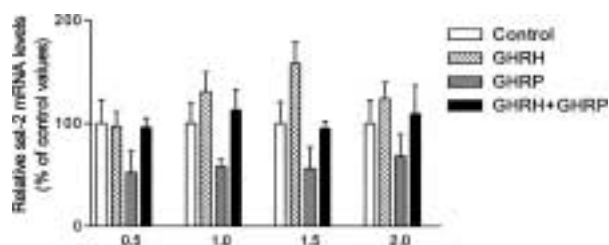


Figure 7 Relative levels of sst-2 mRNAs in ovine pituitary cells in response to treatment with GHRH, GHRP-2 or both. The results are expressed as a percentage of the vehicle-treated control value and represent the means \pm S.E.M. ($n = 4-6$ wells/treatment group). The results are representative of three separate experiments; * $P < 0.05$ compared with the control levels of sst-2 mRNA.

The level of GH mRNA 0.5, 1, 1.5 and 2 h after treatment was greatest in the combined GHRH and GHRP-2 treatment group rather than in the GHRH or GHRP-2 alone treatment groups. Our results are consistent with early reports that 10 nM GHRH treatment of rat pituitary cells in serum-free medium increased GH mRNA expression by 1.8- and almost 2.0-fold at 0.5 and 1 h respectively (40). This demonstrates that the effects of GHRH, GHRP-2 and combinations of GHRH and GHRP-2 on GH mRNA expression are rapid and occur at GH gene transcription level (40).

GHRH and GHRP bind to their specific receptors on the membranes of somatotropes (37). GHRH stimulates GH synthesis by increasing the transcription rate of the GH gene and consequently GH release (40, 41). GHRP-2 enhances pituitary GH gene expression and directly stimulates GH release (42). Our results show that the duration of GHRH or GHRP-2 treatment influences the effects on the corresponding receptor mRNA expression and GH release. GHRH and GHRP treatment for 1.5 h reduces their own receptor mRNA levels. These results are consistent with previous reports which suggest that GHRH in the short-term suppresses its own receptor expression (8, 20). Surprisingly, in this experiment, the short-term GHRH or GHRP treatment (0.5 h) significantly increased the expression of ligand-specific receptor mRNAs, and also increased GH release from ovine pituitary cells. This suggests that GHRH-R or GHS-R mRNA expression may contribute to the increase in GH secretion. GHRH treatment does not significantly influence the mRNA level of GHS-R, nor does GHRP-2 change GHRH-R expression with short-term treatment. The results supported our previous report suggesting that GHRP-2 does not act through the GHRH receptor (6). It is worth mentioning that another study indicated that GHRH, at any dose tested, did not affect GHS-R levels *in vitro* (8).

GH expression is mainly controlled by Pit-1, a member of the homeobox POU (representing a homeo-domain protein family of which the founder members are Pit-1, Oct 1/2 and Unc-86) family of DNA-binding proteins, and GHRH and GHRP-2 elicit a time-dependent activation of Pit-1 expression by anterior pituitary cells (1, 35). Our results indicate that with combined GHRH and GHRP-2 treatment, Pit-1 mRNA expression is increased to 150, 121 and 168% at 0.5, 1.5 and 2 h after treatment respectively. GHRH enhances the levels of Pit-1 mRNA expression 0.5, 1, 1.5 and 2 h after treatment. GHRP-2 also significantly increases the levels of Pit-1 mRNA 0.5 and 2 h after treatment. GHRH and GHRP-2 may activate Pit-1 transcription and stimulates GH expression in pituitary cells through mediation of protein kinase C (PKC), mitogen-activated protein (MAP) kinase and PKA activation (1, 13, 14, 43).

Somatostatin binds to a family of specific receptors and inhibits adenylyl cyclase via G_i proteins, and inhibits GH release but not its biosynthesis (44). In addition, somatostatin may potentially play (dual)

inhibitory and stimulatory roles in controlling GH secretion by acting on two distinct somatotrope cell populations in the porcine pituitary (45). Five somatostatin receptor subtypes have been cloned and characterized and their expression is regulated in a subtype- and tissue-specific manner (46–48). The results of GHRH action on sst receptor synthesis shows that, *in vivo*, a 4 h GHRH infusion and, *in vitro*, a 4 h 10 nM GHRH treatment of rat pituitary cells increased sst-1 and sst-2 mRNA levels but decreased sst-5 mRNA levels (26). In the current study, 10 nM GHRH treatment increased sst-1 mRNA expression 0.5 to 2 h after the treatment. Although GHRH also increased sst-2 mRNA expression this was not statistically significant. This suggests that the acute direct regulatory action of GHRH on the synthesis of sst-1 and sst-2 receptor subtypes may be time-dependent. GHRH-increased sst-1 mRNA expression may be partially due to GHRH-induced increases in Pit-1 mRNA, which activates pituitary sst-1 mRNA expression (14, 49). In contrast, 100 nM GHRP-2 reduced sst-1 and sst-2 mRNA expression 0.5 to 2 h after treatment. GHRP has been suggested to act as a functional SRIF antagonist (11). Inhibition of the SRIF receptors including sst-1 and sst-2 by GHRP-2 supports this view.

In summary, the results of this study indicate that GHRH and GHRP-2 are important mediators regulating GH, GHRH-R, GHS-R, Pit-1, sst-1 and sst-2 mRNA expression, and GH synthesis. Effects on somatotropes manifest as either a priming or an inhibitory modification of the cells, leading to increased or decreased GH secretion. Moreover, the results demonstrate that GHRH and GHRP regulate their receptor synthesis and GH release in a time-dependent manner. This study represents an essential step forward in understanding the influence of GHRH and GHRP on somatotropes. Application of this understanding may aid the development of new GHSs with high efficacy.

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