The effects of GH-releasing hormone/somatostatin on the 5'-promoter activity of the GH gene *in vitro*

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Abstract

The two hypothalamic hormones, GH-releasing hormone (GHRH) and somatostatin (SRIF), are known to regulate GH secretion. However, the effects of these hormones on GH gene expression are not completely clear, partly because of the lack of appropriate host cells maintaining the original characteristics of the somatotroph. Since MtT/S, a pure somatotroph cell line, has become available, the effects of GHRH and SRIF on GH gene transcription have been studied using a subclone of MtT/S (MtT/SGL), in which the GH gene 5'-promoter-luciferase fusion gene was stably incorporated. The expression of GHRH receptor and SRIF receptor subtypes was also studied by RT-PCR. The results showed that MtT/SGL cells intrinsically expressed the functional GHRH receptor and all of the SRIF receptor subtypes. The expression of GHRH receptor was markedly enhanced by glucocorticoid pretreatment and, in the presence of corticosterone and 3-isobutyl-1-methylxanthine, GHRH (at or above 100 pM) stimulated GH gene 5'-promoter activity in a dose-dependent manner. On the other hand, SRIF (100 nM) significantly antagonized the effect of GHRH, which was completely reversed by pretreatment with pertussis toxin (50 ng/ml). Taken together, the present data indicated that both GHRH and SRIF are involved in the transcriptional regulation of the GH gene, and that the effect of SRIF is mediated through pertussis toxin-sensitive G protein. The MtT/SGL cell line is a good *in vitro* model for studying the molecular mechanisms of GH gene transcription by GHRH and/or SRIF.

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Introduction

The synthesis and secretion of growth hormone (GH) in the somatotroph of the anterior pituitary are controlled by multiple regulatory factors (Hartman *et al.* 1993, Harvey 1995, Frohman 1996). Among them, two hypothalamic neuropeptides, GH-releasing hormone (GHRH) and somatostatin (SRIF), are known to be the main regulators of physiological GH release. GHRH binds to the GHRH receptor (GHRH-R) and increases intracellular cAMP, followed by the activation of protein kinase A (Bilezikjian & Vale 1983, Mayo 1992). It also causes an increase in intracellular Ca²⁺, followed by the release of GH (Rawlings *et al.* 1991). In contrast, SRIF is shown to

suppress GH secretion by reducing intracellular cAMP and/or hyperpolarizing the cells through SRIF receptors (sst₁) (Bilezikjian & Vale 1983, Schettini *et al.* 1988, Rawlings *et al.* 1991).

GHRH is known to regulate GH gene expression as well (Barinaga *et al.* 1983), although the detailed molecular mechanisms have not been clarified completely. In previous studies, GH₃ or GC cell lines have been used for studying the transcriptional regulation of the GH gene (Zeytin *et al.* 1984, Shepard *et al.* 1994). However, these cell lines do not express intrinsic GHRH-R, and are thus not suitable for studying the regulation of the GH gene by GHRH.

MtT/S is a somatotroph cell line derived from MtT/F84 rat pituitary tumor cells. Inoue *et al.*

(1990) showed that the cells secrete only GH, not prolactin, and also retain GHRH responsiveness, both of which are characteristics of the pure somatotroph. They also express insulin-like growth factor-I (IGF-I) receptor, and recently we clarified the molecular mechanism of the negative effect of IGF-I on the GH gene 5'-promoter (Niiori-Onishi *et al.* 1999), which is also observed in the normal somatotroph (Yamashita & Melmed 1986, 1987).

In this work we have studied the effects of GHRH/SRIF on the 5'-promoter activity of the GH gene using MtT/S cells. We found that the cell line expresses GHRH-R and all of the subtypes of sst mRNA. Furthermore, our data suggested that SRIF as well as GHRH is involved in the transcriptional regulation of the GH gene in a positive and negative manner respectively.

Materials and methods

Materials

Rat GHRH was a generous gift from Dr S Sawano (Toranomon Hospital, Tokyo, Japan). Somatostatin 14 was purchased from the Peptide Institute (Osaka, Japan). 3-Isobutyl-1-methylxanthine (IBMX), pertussis toxin (PTx) and corticosterone were from Sigma (St Louis, MO, USA).

Cell culture

MtT/SGL, a subclone of the rat somatotroph cell line MtT/S in which the rat GH gene 5'-promoter (≈ 1.75 kb)-luciferase fusion gene is stably incorporated (Niiori-Onishi *et al.* 1999), was used in this study. Cells were maintained in a T₇₅ culture flask with Dulbecco's modified Eagles' medium/F12 culture medium (Life Technologies, Grand Islands, NY, USA) supplemented with 10% horse serum (Sigma), 2.5% fetal bovine serum (Life Technologies) and antibiotics (50 μ U/ml penicillin and 50 μ g/ml streptomycin; Life Technologies) under a 5% CO₂/95% air atmosphere condition at 37 °C. Culture medium was changed twice a week, and the cells were subcultured once a week.

Experiments

MtT/SGL cells pretreated with defined dose(s) of corticosterone (see Results) were plated in poly-D-lysine-coated 3.5 cm diameter culture dishes with

approximately 70% confluence. On the day of each experiment, GHRH or other test reagents, in 1000 $\times$ concentration, or solvent alone, were added directly into the culture medium of each dish, and the cells were incubated for the defined time-interval. Because our preliminary experiments showed that the positive effect of GHRH on the 5'-promoter activity of the GH gene was weak and did not always reach statistical significance, we treated the cells with IBMX (200 μ M; from 30 min before the addition of test reagents to the end of the experiment), to obtain consistent responses. At the end of incubation, the culture medium was removed, and the cells were harvested for the determination of luciferase activity. The luciferase assay was performed as previously described (Aoki *et al.* 1997). IBMX pretreatment did not influence the basal promoter activity of the GH gene (data not shown). When the cAMP responsiveness to GHRH was to be examined, the MtT/SGL cells were pretreated with corticosterone as above for 3 days, and the culture medium was changed to serum-free medium containing IBMX (200 μ M). Cells were then incubated with GHRH (100 nM) for 3 h, and the cAMP concentration in the culture medium was estimated by a specific radioimmunoassay (Yamasa Shoyu, Tokyo, Japan).

RT-PCR

To determine the expression of GHRH-R, total RNAs were isolated from the MtT/SGL cells treated with corticosterone (1, 10 or 100 nM for 3 days) using RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram each of the total RNAs was applied for the RT-PCR (30 cycles) using a one-step RNA PCR kit (Takara Shuzo, Ohtsu, Japan), with a specific primer set for the rat GHRH-R mRNA (Takahashi *et al.* 1995), which amplifies both long and short isoforms of GHRH-R (Mayo 1992, Miller & Mayo 1999). RT-PCR of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA with the same RNAs using a specific primer set (Kimura *et al.* 1998) was carried out as an internal control.

To observe the expression of each subtype of sst mRNA, RT-PCR was carried out as above using 1 μ g total RNAs, either from MtT/SGL cells treated with corticosterone (100 nM) for 3 days, or from the adult rat whole pituitary gland as a control. Specific primer sets for each subtype of sst

mRNA were used (Mori *et al.* 1997), and the cycle number (30 cycles) was determined such that the amplification of GAPDH was submaximal. An RNA sample without reverse transcriptase was also amplified by PCR as a negative control for each reaction. IBMX pretreatment was not carried out in all the RT-PCR experiments.

Data analysis

Samples in each group of the experiments were in triplicate or quadruplicate. All data are expressed as means $\pm$ S.E. When statistical analyses were performed, data were compared by one-way ANOVA with Fisher's protected least significant difference test using the StatView software for Macintosh (version 4.5; SAS institute, Cary, NC, USA), and *P* values below 0.05 were considered significant.

Results

RT-PCR analysis of GHRH-R in MtT/SGL cells

First, we studied whether MtT/SGL cells have intrinsic GHRH-R using the semi-quantitative RT-PCR technique. As shown in Fig. 1, a single band (564 bp) corresponding to mRNA of the short isoform of the rat GHRH-R was amplified with RNA in corticosterone (100 nM)-treated cells. This band was barely detectable in the cells treated with lower doses of corticosterone, although the amplification of GAPDH used as an internal control was comparable among the groups. We could not detect the long splice variant form of GHRH-R (Mayo 1992, Miller & Mayo 1999). Similar results were obtained using RNA from MtT/S cells (data not shown). No band was amplified from samples in which reverse transcriptase was not added (data not shown). These results showed that MtT/SGL cells express the GHRH-R mRNA, which is enhanced by glucocorticoid treatment.

RT-PCR analyses of sst subtypes in MtT/SGL cells

We also examined the expression of sst subtypes in MtT/SGL cells using specific sets of primers for each receptor subtype. As shown in Fig. 2, the bands corresponding to sst₁ (270 bp), sst₂ (304 bp), sst₃ (286 bp), sst₄ (282 bp) and sst₅ (274 bp) were

Figure 1 Expression of the GHRH-R analyzed by RT-PCR in MtT/SGL cells. The figure shows photographs of the ethidium bromide-stained products using agarose gel electrophoresis. cDNA produced from an RT reaction using total RNA from MtT/SGL cells treated with three doses of corticosterone for 3 days was amplified using PCR (30 cycles) with pairs of oligonucleotide primers specific for rat GHRH-R. A DNA fragment compatible with the short isoform of GHRH-R (564 bp) was intensely amplified from the cells treated with 100 nM corticosterone, whereas it was barely detectable in the cells treated with lower doses of corticosterone. The amplification of GAPDH (used as an internal control) was comparable among the groups.

amplified. No band was amplified when samples were treated without reverse transcriptase (data not shown). In the whole pituitary gland used as a control, sst₂, sst₃ and sst₅ were amplified under the same RT-PCR condition.

Effect of corticosterone pretreatment on GHRH responsiveness of cAMP production

We then studied the effect of GHRH on cAMP generation in MtT/SGL cells pretreated with various doses of corticosterone. As shown in Fig. 3A, GHRH significantly stimulated cAMP efflux into culture medium when the cells were pretreated with corticosterone (100 nM for 3 days), corresponding to the positive effect of corticosterone on GHRH-R expression in Fig. 1. In contrast, no effect of GHRH was observed under low doses of corticosterone (1–10 nM).

Effect of corticosterone pretreatment on GHRH responsiveness of the GH gene 5'-promoter activity

We also studied the effect of GHRH on the transcriptional activity of the GH gene in

Figure 2 Expression of the sst subtypes analyzed by RT-PCR in the adult rat whole pituitary gland (A) and in MtT/SGL cells (B). The Figure shows photographs of the ethidium bromide-stained products using agarose gel electrophoresis. cDNA produced from an RT reaction using total RNA from either MtT/SGL cells or the rat whole pituitary gland was amplified using PCR (30 cycles) with pairs of oligonucleotide primers specific for each subtype of the rat ssts. DNA fragments with the predicted lengths for sst₂, sst₃ and sst₅ were amplified from the whole pituitary gland, whereas all the subtypes were amplified from MtT/SGL cells. M, molecular size marker.

MtT/SGL cells pretreated with the three doses of corticosterone. As shown in Fig. 3B, GHRH significantly stimulated the 5'-promoter activity of the GH gene when cells were pretreated with corticosterone (100 nM for 3 days) and IBMX (200 μ M; from 30 min before the addition of GHRH), again corresponding to the positive effect of corticosterone on GHRH-R expression (data shown above). GHRH had no effect on the cells with low doses of corticosterone (1–10 nM) (Fig. 3A and B) or without pretreatment (data not shown). Subsequent experiments concerning the effect of GHRH on the 5'-promoter activity of the GH gene were therefore carried out using MtT/SGL cells pretreated with 100 nM corticosterone for 3–4 days.

Time-course and dose-response effects of GHRH on the 5'-promoter activity of the GH gene

We further characterized the effect of GHRH on the transcriptional regulation of the GH gene

Figure 3 The effect of corticosterone pretreatment on the GHRH responsiveness of cAMP generation and the GH 5'-promoter activity in MtT/SGL cells. (A) Cells were pretreated with 1, 10 or 100 nM of corticosterone for 3 days, and then incubated with GHRH (100 nM) for 3 h in the presence of IBMX (200 μ M; from 30 min before the addition of GHRH to the end of the experiment). cAMP efflux into the culture medium was determined by the cAMP assay. (B) Cells were pretreated as above, and then incubated with GHRH (100 nM) for 8 h in the presence of IBMX (200 μ M). Open and closed bars represent the control (C) and GHRH-treated groups respectively. * $P < 0.05$ vs control value.

under pretreatment with corticosterone (100 nM for 3 days) and IBMX (200 μ M; from 30 min before the addition of GHRH). The time-course study showed that GHRH (100 nM) stimulated the GH gene 5'-promoter activity in a time-related manner (Fig. 4A). A significant effect occurred at 4 h, and the maximal effect was observed 8 h after the addition of GHRH, with an approximately twofold increase in the transcriptional activity compared with the value at time zero. The dose-response study also showed that an 8-h treatment with GHRH had a dose-dependent positive effect on GH 5'-promoter activity; the

Figure 4 The effects of GHRH on the GH 5'-promotor activity in MtT/SGL cells. (A) The time-course effect; cells were incubated with corticosterone (100 nM) for 3 days, and then treated with GHRH (100 nM) for 0–12 h in the presence of IBMX (200 μ M; from 30 min before the addition of GHRH to the end of experiment). (B) The dose-response effect; cells were pretreated as above, and also with five different doses of GHRH (100 pM to 1 μ M) for 8 h. C, control. * $P < 0.05$ vs control value or value at time zero.

minimal effect was observed at 100 pM, and the maximal one at or above 1 nM (Fig. 4B). IBMX alone had no effect on the basal promoter activity of the GH gene (data not shown).

Combined effects of GHRH and SRIF on the 5'-promoter activity of the GH gene

Finally, we tested the combined effects of GHRH and SRIF on the transcriptional activity of the GH gene. GHRH alone (100 nM) again significantly stimulated the GH 5'-promoter activity. The addition of SRIF (100 nM), however, partially but significantly suppressed the GHRH-induced transcriptional activity of the GH gene (Fig. 5A), despite the fact that there was no effect with SRIF alone (data not shown). This inhibition was eliminated when the same experiment was carried out under the treatment with PTx (from 8 h before GHRH/SRIF treatment to the end of the experiment), suggesting that SRIF exerts its negative effect through PTx-sensitive G protein.

Discussion

We have studied here the effect of GHRH and/or SRIF on the GH gene using the pure somatotroph cell line MtT/S. We have confirmed that GHRH stimulates the 5'-promoter activity of the gene. Furthermore, we found that SRIF is involved in the regulation of the GH gene in such a way that the hormone interferes with the effect of GHRH through PTx-sensitive G protein.

The transcriptional regulation of the GH gene has been examined in previous studies using GH₃, GC or GH₄C₁ cells (Lefevre *et al.* 1987, Brent *et al.* 1989). The GH₃ and GC cell lines, however, are somatomammotrophs which express both GH and prolactin, the GH₄C₁ cells being a mammotroph which secretes only prolactin, and thus all of them may be different from the pure somatotroph in nature. Moreover, neither GH₃ nor GC cells possess GHRH responsiveness, implying that they are not suitable for the examination of the physiological effect of GHRH (Chen *et al.* 1998,

Figure 5 The combined effects of GHRH and SRIF on the GH 5'-promoter activity in MtT/SGL cells. (A) Cells were pretreated with corticosterone (100 nM) for 4 days, and then treated with GHRH (100 nM) alone, or GHRH and SRIF (100 nM) for 8 h in the presence of IBMX (200 μ M; from 30 min before the addition of GHRH to the end of experiment). (B) Cells were pretreated as (A) and also with PTx (50 ng/ml; from 8 h before the addition of GHRH/SRIF to the end of experiment). The cells were then treated in the same manner as in (A). C, control. * $P < 0.05$ vs control value.

Lee *et al.* 2001). The MtT/S cell line, in contrast, has been shown to express GH alone, not prolactin, and it maintains GHRH responsiveness (Inoue *et al.* 1990). Expression of GHRH-R mRNA in MtT/S cells has already been reported (Nogami *et al.* 1999, Voss *et al.* 2001), and we also confirmed the expression of all of the subtypes of ssts. Altogether, MtT/S seems to be an appropriate homologous cell line for studying the transcriptional regulation of the GH gene by GHRH and/or SRIF.

GHRH is shown to play a pivotal role in the regulation of GH gene expression in both *in vivo* and *in vitro* systems (Hartman *et al.* 1993, Harvey 1995, Frohman 1996). In this study, we clearly showed the positive effect of GHRH on the transcription of the GH gene. Furthermore, the effect was dose-dependent, and occurred at physiological concentrations such as 100 pM GHRH in the hypophyseal portal vein. GHRH stimulation on the amount of GH mRNA or its promoter activity has been demonstrated previously using primary culture, somatomammotroph GH₃ or non-endocrine CV1/F9 cell lines (Barinaga *et al.* 1983, Cohen *et al.* 1999). We have also

confirmed the positive effect of GHRH on GH gene transcription through intrinsic GHRH-R in the pure somatotroph cell line MtT/S. The increment in the promoter activity, however, was at most approximately twofold even in the presence of IBMX in this cell line, which appears to be the reason why the effect was detectable not by the changes in the amount of GH mRNA (Voss *et al.* 2001) but by a sensitive reporter gene assay which reflects the *de novo* changes in the 5'-promoter activity of the GH gene.

It is of interest that the GHRH responsiveness was glucocorticoid dependent; the effect was recognized only after the cells were treated with 100 nM corticosterone, a physiological rat glucocorticoid, for 3 or more days. This treatment caused both the expression of GHRH-R and cAMP responsiveness to GHRH, suggesting that the restoration of GHRH responsiveness is mediated, at least in part, through the increment in functional GHRH-R. The positive effect of glucocorticoid on GHRH-R has been shown in both *in vitro* and *in vivo* studies (Tamaki *et al.* 1996, Miller & Mayo 1997), and also in MtT/S cells

(Miller *et al.* 1999, Nogami *et al.* 1999). In addition, Miller *et al.* (1999) showed that long and short isoforms of GHRH-R mRNA are expressed in MtT/S cells. They also found that the bio-inactive long isoform is dominant in the cells with an adhesive nature, a subpopulation of the cells in culture. However, RT-PCR analysis showed that only the bioactive short form of the receptor was amplified in both the MtT/S and MtT/SGL cell lines used in this study, and thus the cell line is thought to possess the potency to respond to GHRH through bioactive GHRH-R. The reason why our cells did not express the long isoform is currently unknown, but was possibly due to the clonal difference of the cell line.

We also examined the effect of SRIF on the transcriptional activity of the GH gene in this study. Previous works have shown that SRIF is involved in the regulation of GH secretion, but not of GH gene expression (Barinaga *et al.* 1985). More recent work *in vivo*, however, has shown controversial data, maintaining the debate on this issue (Sugihara *et al.* 1993). Our data presented in this paper clearly showed that SRIF partly but significantly suppressed GHRH-induced GH gene transcription, although SRIF alone did not influence the basal promoter activity. Furthermore, the negative effect was completely eliminated by pretreatment with PTx, an inhibitor of suppressive G protein (G_i or G_o). Thus, SRIF is involved in the regulation of GH gene transcription as well as GH secretion, by interfering with the positive effect of GHRH. This effect seems to be mediated through some subtype(s) of sst, because RT-PCR analysis showed the expression of all of the sst subtypes (sst₁–sst₅) in this cell line, which includes the dominant subtypes (sst₂ and sst₅) expressed in the normal somatotroph *in vivo* (O'Carroll & Krempels 1995). We hypothesize that SRIF inhibits the GH gene at the adenylate cyclase level through sst(s) and inhibitory G protein (Bilezikjian & Vale 1983). Alternatively, SRIF exerts its effect downstream of cAMP by inhibiting the non-selective cation channel or calcium channel through G protein/potassium channel-mediated hyperpolarization, which has been shown in somatotroph cells (Chen *et al.* 1994). The issue is currently under investigation in our laboratory.

In conclusion, the present data suggest that both GHRH and SRIF play an important role in the transcriptional regulation of the GH gene. Taken

together with our previous data (Niiori-Onishi *et al.* 1999), we assume that hypothalamic SRIF exerts an acute inhibitory effect by eliminating GHRH stimulation, and IGF-I exerts a chronic inhibitory effect through a long negative feedback loop. Further studies using our homologous MtT/SGL cell line will clarify the intracellular cross-talk among the second messenger systems linked with GHRH/SRIF/IGF-I.

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