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

M Morishita, Y Iwasaki¹, A Onishi, M Asai, N Mutsuga, M Yoshida, Y Oiso, K Inoue² and T Murohara

Department of Medicine, Nagoya University Graduate School of Medicine and Hospital, Nagoya 466-8550, Japan
¹Department of Clinical Pathophysiology, Nagoya University Graduate School of Medicine and Hospital, Nagoya 466-8550, Japan
²Department of Cell Regulation, Saitama University, Saitama 338-0825, Japan

(Requests for offprints should be addressed to Y Iwasaki, Department of Clinical Pathophysiology, Nagoya University Graduate School of Medicine and Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan; Email: iwasakiy@med.nagoya-u.ac.jp)

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.

Journal of Molecular Endocrinology (2003) 31, 441–448

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 (ssts) (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 µU/ml penicillin and 50 µg/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-n-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 × 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 µM; 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 µM). 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 µg 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 ± 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 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
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 µM; 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 under pretreatment with corticosterone (100 nM for 3 days) and IBMX (200 µM; 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
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 (Lefèvre 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,
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 nM 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, somatotroph 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.
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.

Acknowledgements

We are indebted to Dr Shinji Sawano for the generous gift of rat GHRH. This work was supported in part by a research grant from the Foundation for Growth Science, Tokyo, Japan.

References


