IGF-I regulates pro-opiomelanocortin and GH gene expression in the mouse pituitary gland

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Abstract

IGF-I is expressed in somatotrophs, and IGF-I receptors are expressed in most somatotrophs and some corticotrophs in the mouse pituitary gland. Our recent study demonstrated that IGF-I stimulates the proliferation of corticotrophs in the mouse pituitary. These results suggested that somatotrophs regulate corticotrophic functions as well as somatotrophic functions by the mediation of IGF-I molecules. The present study aimed to clarify factors regulating pituitary IGF-I expression and also the roles exerted by IGF-I within the mouse anterior pituitary gland. Mouse anterior pituitary cells were isolated and cultured under serum-free conditions. GH (0.5 or $1 \mu\text{g/ml}$), ACTH (10^{-8} or 10^{-7} M), GH-releasing hormone (GHRH; 10^{-8} or 10^{-7} M), dexamethasone (DEX; 10^{-8} or 10^{-7} M) and estradiol- 17β (E2; 10^{-11} or 10^{-9} M) were given for 24 h. IGF-I mRNA levels were measured using competitive RT-PCR, and GH and pro-opiomelanocortin (POMC) mRNA levels were measured using Northern blotting analysis. GH treatment significantly increased IGF-I mRNA levels (1.5- or 2.1-fold).

ACTH treatment did not alter GH and IGF-I mRNA levels. IGF-I treatment decreased GH mRNA levels (0.7- or 0.5-fold), but increased POMC mRNA levels (1.8-fold). GH treatment (4 or $8 \mu\text{g/ml}$) for 4 days increased POMC mRNA levels. GHRH treatment increased GH mRNA levels (1.3-fold), but not IGF-I mRNA levels. DEX treatment significantly decreased IGF-I mRNA levels (0.8-fold). E2 treatment did not affect IGF-I mRNA levels. GH receptor mRNA, probably with GH-binding protein mRNA, was detected in somatotrophs, and some mammotrophs and gonadotrophs by *in situ* hybridization using GH receptor cDNA as a probe. These results suggested that IGF-I expression in somatotrophs is regulated by pituitary GH, and that IGF-I suppresses GH expression and stimulates POMC expression at the transcription level. Pituitary IGF-I produced in somatotrophs is probably involved in the regulation of somatotroph and corticotroph functions.

Journal of Endocrinology (2003) **178**, 71–82

Introduction

Insulin-like growth factor-I (IGF-I) is a 70 amino acid polypeptide that is produced in a number of tissues. Several reports have shown that IGF-I regulates the proliferation and differentiation of various cells in an autocrine and/or paracrine manner. Pituitary cells synthesize IGF-I (Fagin *et al.* 1988, Gonzalez-Parra *et al.* 2001), and express IGF-I receptors (Bach & Bondy 1992). Our previous study showed that IGF-I is expressed in somatotrophs, and that type I receptors for IGF-I are expressed in somatotrophs and some corticotrophs in the mouse pituitary (Honda *et al.* 1998). We recently demonstrated that IGF-I stimulated the proliferation of anterior pituitary cells, in particular corticotrophs, indicating that the proliferation of anterior pituitary cells was stimulated by IGF-I produced in the anterior pituitary cells (Oomizu *et al.* 1998). In addition, there are many reports showing that IGF-I regulates GH expression and secretion at the

pituitary level (Goodyer *et al.* 1984a, Yamashita & Melmed 1986) and/or the hypothalamic level (Abe *et al.* 1983, Tannenbaum *et al.* 1983). These findings suggested that pituitary IGF-I regulates the functions of corticotrophs and somatotrophs. However, there are few reports about the effects of IGF-I on adrenocorticotrophic hormone (ACTH) secretion, although Goodyer *et al.* (1984a) reported that ACTH release remained unaltered with IGF-I treatment. Therefore, the aim of the present study was to clarify the role of IGF-I on mouse pituitary corticotrophs as well as somatotrophs.

It is necessary to study the regulatory mechanism for pituitary IGF-I expression in order to understand the physiological roles of IGF-I within the pituitary gland. Pituitary IGF-I synthesis is thought to be dependent on growth hormone (GH), based on reports that pituitary IGF-I gene expression is enhanced in GH-secreting tumor-bearing rats relative to control animals (Fagin *et al.* 1988), and that GH treatment increases IGF-I mRNA

levels in pituitary tumor GH₃ cells (Fagin *et al.* 1989). In the present study, we studied the effects of GH, estradiol-17 β (E2), ACTH, GH-releasing hormone (GHRH) and dexamethasone (DEX) treatment on IGF-I expression in mouse pituitary cells *in vitro*. We used a competitive RT-PCR assay with heterologous sequence cDNA competitors to measure the low levels of IGF-I mRNA expression in cultured mouse pituitary cells. GH receptor mRNA-expressing cells were analyzed by non-radioisotopic *in situ* hybridization, and their hormone content was analyzed by immunofluorescence microscopy.

In this study, we have demonstrated a stimulatory effect of IGF-I on pro-opiomelanocortin (POMC) gene expression and an inhibitory effect on GH gene expression in mouse pituitaries. Somatotrophs are the main source of pituitary IGF-I. We have also shown a stimulatory effect of GH on IGF-I gene expression. These findings provide insights into the interaction between somatotrophs and corticotrophs mediated by IGF-I that may be one example of an intrapituitary regulatory system.

Materials and Methods

Animals

Male ICR mice (CLEA Japan Inc., Osaka, Japan) were kept in a temperature-controlled animal room with free access to commercial diet CE-7 (CLEA Japan Inc.) and tap water *ad libitum*. All animal care and experiments were carried out according to the Guidelines for Animal Experimentation, Faculty of Science, Okayama University, Japan, and the NIH Guidelines for the Care and Use of Laboratory Animals.

Culture

Anterior pituitaries from 2-month-old male mice were dissociated with trypsin (0.5% (w/v); DIFCO, Detroit, MI, USA) as previously described (Oomizu *et al.* 1998). Cell viability was checked using the trypan blue exclusion test and was usually found to be more than 95%. The isolated pituitary cells were suspended in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) without phenol red (Sigma Chemical Co., St Louis, MO, USA) containing heat-inactivated fetal calf serum (10% (v/v); Gibco BRL, Life Technologies Inc., Rockville, MD, USA). The cells were seeded on poly-L-lysine (Sigma Chemical Co.)-coated 6-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ, USA) at a density of 1×10^6 cells/2 ml per well for RNA measurement, and on poly-L-lysine-coated glass coverslips (diameter 13 mm; Matsunami Glass Ind., Osaka, Japan) in 24-well tissue culture plates (Becton

Dickinson) at a density of 1×10^5 cells/1 ml per well for the detection of DNA synthesis. These cells were cultured in DMEM/F12 medium containing 10% fetal calf serum for 1 day, and in serum-free DMEM/F12 medium containing 100 mg/l hydrocortisone, 400 ng/l 3,3'-tri-iodothyronine, 10 mg/l transferrin, 10 ng/l bovine glucagon, 200 ng/l parathyroid hormone and 5 mg/l sodium selenite for 3 days. All supplements were obtained from Sigma Chemical Co. Culturing was performed in a humidified incubator with 5% CO₂ and 95% air at 37 °C. The culture was allowed to proceed in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Hormone treatment

After a 3-day culture in serum-free DMEM/F12 medium, cultured cells were treated with rat GH (0.5 or 1 μ g/ml; NIDDK-rGH-B-11; NIH, Bethesda, MD, USA), rat ACTH (10^{-8} or 10^{-7} M; Sigma Chemical Co.), rat GHRH (10^{-8} or 10^{-7} M; Sigma Chemical Co.), E2 (10^{-11} or 10^{-9} M; Sigma Chemical Co.), DEX (10^{-8} or 10^{-7} M; Sigma Chemical Co.), and human recombinant IGF-I (7.5 or 75 ng/ml; Amersham Pharmacia Biotech, Uppsala, Sweden) for 24 h followed by RNA extraction.

In a separate experiment, cultured cells were treated with rat GH (4 or 8 μ g/ml; NIDDK-rGH-B-11) for 4 days followed by RNA extraction. The medium supplemented with GH was exchanged every 2 days during the hormone treatment.

Primers and competitors

Primers for mouse IGF-I, mouse GH receptor and mouse β -actin were designed based on previous reports (Smith *et al.* 1988, Ho *et al.* 1995, Matsuda & Mori 1997). The sequences of the primers are as follows: IGF-I 5' sense primer; 5'-GGACCAGAGACCCTTTGCGGGG-3', IGF-I 3' antisense primer; 5'-GGCTGCTTTTGT AGGCTTCAGTGG-3', GH receptor 5' sense primer; 5'-TTAGTTTGACCGGGATTCGTGG-3', GH receptor 3' antisense primer; 5'-AATCTTTGGAAGTGGGA CTGGG-3', β -actin 5' sense primer; 5'-TCTAGACTT CGAGCAGGAGATGGCC-3', β -actin 3' antisense primer; 5'-CTAGAAGCACTTGCAGGTGCACGATG-3'. These primers were expected to generate cDNAs of 210, 437 and 471 bp for IGF-I, GH receptors and β -actin respectively. All primers were synthesized by Gibco BRL Custom Primers (Life Technologies Oriental, Inc., Tokyo, Japan).

Competitors of IGF-I and β -actin were constructed using a competitive DNA construction kit (Takara Shuzo Co. Ltd, Otsu, Japan). The competitors contained heterologous λ DNA sequences including primer sequences of IGF-I or β -actin at both ends. Using the

competitors, the primers of IGF-I and β -actin generated PCR products of 346 and 550 bp respectively.

Competitive RT-PCR

Total RNA was prepared from the cultured pituitary cells using the method of Chomczynski & Sacchi (1987). Total RNA (1 μ g) in a final volume of 20 μ l was subjected to RT reaction using a SuperScript preamplification system for first strand cDNA synthesis (Life Technologies Oriental, Inc.) with oligo(dT)_{12–18} primers according to the manufacturer's instructions. In all RNA samples without the RT reaction, no amplified products were detected in the following PCR amplification, indicating no contamination of genomic DNA (data not shown).

A competitive RT-PCR assay was developed according to a previously established method (Kirk *et al.* 1994). One microliter of the RT samples from the cultured pituitary cells was used in one reaction of competitive RT-PCR with a series of dilutions of the competitors (for IGF-I, 10^5 , 5×10^5 and 10^6 copies; for β -actin, 10^7 , 5×10^7 and 10^8 copies). The competitive RT-PCR for GH-treated and E2-treated samples was carried out using AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA) and a thermal cycler, Gene Amp PCR System 9600 (Applied Biosystems). The conditions for the PCR were as follows: after activation of the DNA polymerase by incubating for 9 min at 95 °C, 40 cycles of reactions including denaturation for 30 s at 95 °C and extension for 1 min at 60 °C were carried out, followed by additional extension for 10 min at 60 °C. The competitive RT-PCR for ACTH-treated, GHRH-treated and DEX-treated samples was carried out using TaKaRa Taq (Takara Shuzo Co. Ltd) and a thermal cycler, Gene Amp PCR System 9700 (Applied Biosystems). The conditions for the PCR were as follows: incubating for 10 s at 95 °C, 40 cycles of reactions including denaturation for 30 s at 95 °C and extension for 1 min at 60 °C were carried out, followed by additional extension for 10 min at 60 °C.

Ten microliters of PCR product were electrophoresed on 2.0% (w/v) agarose gel, stained with ethidium bromide, photographed under ultraviolet illumination, and compared with a known standard, 100 bp DNA ladder (Life Technologies Oriental Inc.) for size determination. The band intensities were quantified by NIH Image (version 1.61). The log ratio between the band intensity of RT samples and that of the competitor was plotted against the concentrations of the competitors. The amount of the competitors required to give a 1:1 molar ratio of sample and competitor PCR products was then determined graphically. This gives a measure of IGF-I mRNA levels contained in the samples. β -Actin mRNA levels were used as an internal control according to previous studies (Fan *et al.* 1995, Chesnokova & Melmed 2000, Fiorentini *et al.* 2002), and IGF-I mRNA levels were normalized relative to the amount of β -actin mRNA.

Northern blotting analysis

Northern blotting analysis was carried out according to the method described by Takeuchi *et al.* (1988) with slight modifications. Six micrograms of total RNA, prepared from the cultured pituitary cells using the method of Chomczynski & Sacchi (1987), were denatured at 65 °C for 15 min in 50% (v/v) formamide–6.5% (w/v) formaldehyde–MOPS buffer (20 mM 3-(N-morpholino)–2-hydroxypropanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA, pH 7.0), and electrophoresed in a 6.5% (w/v) formaldehyde–1% (w/v) agarose gel in MOPS buffer. The RNA was electrophoretically transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech) at 4 °C overnight.

Mouse POMC cDNA (pcrmPOMC-JH1) and mouse GH receptor cDNA clone (pmghr1–1), both obtained by RT-PCR and subcloned into a vector pGEM3Zf (+) and sequenced in our laboratory, rat GH cDNA (kindly supplied by Dr J A Martial, University of Liège, Belgium) and mouse β -actin cDNA (Ambion Inc., Austin, TX, USA) were radioisotopically labeled with α -³²P-dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) by a random primer DNA labeling kit version 2.0 (Takara Shuzo Co. Ltd).

The membranes were hybridized at 43 °C for 16 h in a solution containing 5 $\times$ SSPE, 1 $\times$ Denhardt's solution, 50% (v/v) deionized formamide, 20 μ g/ml denatured herring sperm DNA and labeled cDNA probe. The membranes were washed in 5 $\times$ SSC and 0.1% (w/v) SDS and 1 $\times$ SSC and 0.1% (w/v) SDS at 45 °C for 30 min, followed by a final wash in 0.1 $\times$ SSC and 0.1% (w/v) SDS at 45 °C for 30 min. They were exposed to RX-U (Fuji medical X-ray film; Fuji Photo Film, Tokyo, Japan) at –80 °C with double intensifying screens overnight. As an internal control, β -actin mRNA was detected with β -actin cDNA probe after the detection of GH or POMC mRNAs. The signal intensities were normalized relative to the intensity of β -actin mRNA.

In situ hybridization

GH receptor mRNA-expressing cells were detected by non-radioisotopic *in situ* hybridization as described previously (Honda *et al.* 1998). Pituitary glands were quickly fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.6) at 4 °C overnight. They were then embedded in paraffin, and sectioned at a thickness of 5 μ m. The sections were digested with 10 mg/ml proteinase K (Merck, Darmstadt, Germany) at 37 °C for 30 min and the reaction of proteinase K was blocked with 0.2% (w/v) glycine in 0.01 M PBS. The sections were postfixed with 4% paraformaldehyde in 0.01 M PBS, and washed twice with PBS for 30 s. Finally, the sections were incubated in 0.25% acetic anhydride–0.1 M triethanolamine for 10 min at room temperature.

Preparation and detection of cDNA probes was carried out using a digoxigenin (DIG) DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany). Mouse GHR cDNA (pmghr1-1) was labeled with DIG-11-UTP by a random primer method at 37 °C for 20 h. pBR328 plasmids were labeled and used as a negative control for *in situ* hybridization. After the random-labeling reaction, DIG-labeled DNA probes were purified as follows. Twenty microliters labeling mixture were added to 2 µl 5 M LiCl, 3 µl 10 mg/ml herring sperm DNA and 75 µl prechilled ethanol, and kept at -80 °C for at least 30 min, followed by centrifugation (11 000 g, 4 °C, 20 min) to precipitate only the DIG-labeled DNA probes.

The pituitary sections were placed in a moist chamber and hybridized at 42 °C overnight in a solution containing 5 × SSPE, 1 × Denhardt's solution, 10% (w/v) sodium dextran sulfate, 50% (v/v) deionized formamide, 120 µg/ml denatured herring sperm DNA and 0.3 ng/µl DIG-labeled probe. The slides were washed in 1 × SSC at room temperature for 10 min, then in 1 × SSC at 45 °C for 15 min, then in 0.5 × SSC at 45 °C for 15 min. The final wash was carried out in 0.2 × SSC at room temperature for 3 min. Non-specific binding was blocked with 0.5% (w/v) bovine serum albumin in TN buffer (0.1 M Tris-HCl, 0.3 M NaCl, pH 7.5) for 30 min. Anti-DIG-alkaline phosphatase conjugate (1:1000) was applied to the sections at room temperature for 1 h. The slides were washed three times with TN buffer containing 0.3% Tween 20 for 10 min. The hybridization signal was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens.

Immunocytochemical analysis of GH receptor mRNA-expressing cells

After the *in situ* hybridization analysis of GH receptor mRNA, the sections were immunocytochemically stained with one of the polyclonal antisera raised against anterior pituitary hormones: rabbit anti-pig ACTH (1:2000; Advance, Tokyo, Japan), rabbit anti-rat thyrotropin β (TSHβ; 1:2000, anti-rβTSH-IC-1; NIDDK), guinea pig anti-rat luteinizing hormone β (LHβ; 1:2500; anti-rβLH-IC-2; NIDDK), guinea pig anti-rat follicle-stimulating hormone β (FSHβ; 1:2000, anti-rβFSH-IC-1; NIDDK), rabbit anti-rat GH, (1:2000; Takahashi 1992), and rabbit anti-mouse prolactin (PRL; 1:2000; Shikibo, Kusatsu, Japan). The primary antibodies were localized with anti-rabbit IgG, anti-monkey IgG or anti-guinea pig IgG fluorescein-labeled antisera for 30 min. Immunostaining was abolished by omission of each primary antibody or by the use of primary antibodies (working dilution, 100 µl) preabsorbed with 10 µg of each highly purified pituitary hormone (rat GH, rat PRL, rat ACTH, rat TSH, rat LH, rat FSH respectively) at 4 °C for 24 h. The specificity of antibodies to GH and PRL was also checked with the immunoblotting assay (Takahashi 1992). The pituitary

cells were incubated with one of the antibodies to GH, PRL, ACTH or TSHβ at room temperature for 2 h or one of the antibodies to LHβ or FSHβ at 4 °C for 48 h. The primary antibodies were localized with fluorescein-labeled antibodies (EY Laboratories, San Mateo, CA, USA).

Statistical analysis

Statistical significance of the difference between the means was assessed with one-way analysis of variance. Three independent experiments were performed in each study.

Results

Competitive RT-PCR assay for IGF-I mRNA

Aliquots of the RT products (equivalent to 0.2, 1.0 and 2.0 µl of the RT products) were subjected to PCR, and the amplifications were carried out with IGF-I competitors (10^5 to 10^6 copies) and β-actin competitors (10^7 to 10^8 copies). The ethidium bromide-stained gels were quantified, and the ratios of the band intensities of the PCR products from the RT products to the band intensities of the PCR products from the competitor template were calculated. We verified that the amount of PCR products increased in accordance with the increase in the amount of RT products used as the template for PCR in the determination of the mRNA levels of IGF-I and β-actin (data not shown). These results indicated that the competitive RT-PCR assay allowed us to semi-quantitatively estimate the IGF-I and β-actin mRNA levels.

Effect of GH, ACTH, GHRH, DEX and E2 treatment on IGF-I mRNA expression

IGF-I mRNA levels in the cultured cells were quantified using the competitive RT-PCR assay (Fig. 1). GH treatment (1 µg/ml) significantly increased IGF-I mRNA levels 2.1-fold over the control level ($P < 0.01$). ACTH treatment (10^{-8} and 10^{-7} M) and GHRH treatment (10^{-8} and 10^{-7} M) did not change the IGF-I mRNA levels. DEX treatment (10^{-7} M) significantly decreased IGF-I mRNA levels 0.8-fold ($P < 0.05$). Since E2 treatment is known to stimulate IGF-I expression in the mouse uterus, it was investigated. However, E2 treatment (10^{-11} and 10^{-9} M) did not change the IGF-I mRNA levels.

Effect of GH, IGF-I, ACTH, GHRH and DEX treatment on GH mRNA expression

GH treatment (0.5 and 1 µg/ml) did not significantly change GH mRNA expression (Fig. 2). IGF-I treatment

Figure 1 Effect of GH, ACTH, GHRH, DEX and E2 treatment on IGF-I expression. Cultured pituitary cells were treated with GH (0.5 and 1 μg/ml), ACTH (10⁻⁸ and 10⁻⁷ M), GHRH (10⁻⁸ and 10⁻⁷ M), DEX (10⁻⁸ and 10⁻⁷ M) and E2 (10⁻¹¹ and 10⁻⁹ M) for 24 h. IGF-I mRNA levels were measured by competitive RT-PCR. (A) Typical example of ethidium bromide-stained competitive RT-PCR products (upper panel, IGF-I; lower panel, β-actin). In each hormone treatment, 10⁶ or 10⁸ (lanes 1, 4 and 7), 5 × 10⁵ or 5 × 10⁷ (lanes 2, 5 and 8) and 10⁵ or 10⁷ copies (lanes 3, 6 and 9) of IGF-I or β-actin competitors were used. (B) Summary of the competitive RT-PCR analysis of IGF-I mRNA levels. The values represent the relative levels of IGF-I mRNA compared with the level in control cells without hormone treatment which was defined as 1.0. IGF-I mRNA levels were normalized relative to the amount of β-actin mRNA. Each value is the mean ± S.E.M. of three different preparations of RNA. **P* < 0.05, ***P* < 0.01 compared with control.

(7.5 and 75 ng/ml) significantly decreased GH mRNA levels 0.7- and 0.5-fold respectively (*P* < 0.05; Fig. 3). ACTH treatment did not change GH mRNA levels. GHRH treatment (10⁻⁸ and 10⁻⁷ M) significantly increased GH mRNA levels 1.3-fold over control levels at both concentrations (*P* < 0.05). DEX treatment (10⁻⁸ and 10⁻⁷ M) did not change GH mRNA levels.

Effect of IGF-I and GH treatment on POMC mRNA expression

IGF-I treatment (75 ng/ml) for 24 h significantly increased POMC mRNA levels 1.8-fold (*P* < 0.05), but treatment with a low concentration of IGF-I (7.5 ng/ml) did not change POMC expression (Fig. 4). GH treatment (4 and 8 μg/ml) for 4 days significantly increased POMC mRNA levels (*P* < 0.05; Fig. 5).

Northern blotting analysis of GH receptor mRNA and GH-binding protein (GHBP) mRNA

Using a cDNA probe (pmghr1-1) encoding the common extracellular domain of the GH receptor and the circulating GHBP, Northern blotting analysis revealed two bands (1.4 and 4.2 kb) in the RNA samples obtained from the anterior pituitary glands of male mice (Fig. 6). The 1.4 kb band is considered to be GHBP mRNA and the 4.2 kb band is considered to be GH receptor mRNA.

In situ hybridization analysis of GH receptor mRNA-expressing cells

GH receptor mRNA in mouse pituitary glands was detected by non-radioisotopic *in situ* hybridization. GH receptor mRNA was expressed in a subpopulation of

Figure 2 Effect of GH treatment on the expression of the GH gene in mouse pituitary cells in primary culture. (A) Autoradiogram of the results of a Northern blotting analysis of RNA prepared from pituitary cells treated with 0.5 or 1.0 μg rat GH per well (1 ml) for 24 h. The upper band indicates GH mRNA, and the lower band indicates β-actin mRNA. (B) Summary of Northern blotting analysis of GH mRNA levels. The values represent the relative level of GH mRNA compared with the level in control cells without GH treatment which was defined as 1.0. In each sample, GH mRNA levels were normalized relative to the amount of β-actin mRNA. Each value is the mean $\pm$ S.E.M. of three different preparations of RNA.

secretory cells in the anterior lobe (Fig. 7A). GH receptor mRNA was not detected in the intermediate and posterior lobes. The cells expressing GH receptor mRNA were round or oval in shape, medium-sized and accounted for approximately 50% of all anterior pituitary cells in the adult male mice (Fig. 7B). GH receptor mRNA-expressing cells were distributed throughout the anterior pituitary gland. In the control experiments for the *in situ* hybridization, no signal was detected with DIG-labeled pBR328 as the probe (Fig. 7C).

Characterization of GH receptor mRNA-expressing cells in the anterior pituitary

The anterior pituitary hormones produced in the GH receptor mRNA-expressing cells were determined immunocytochemically. The GH receptor mRNA-expressing cells contained immunoreactive GH (Fig. 8A and B). In some of the mammotrophs and FSHβ-immunoreactive gonadotrophs, GH receptor mRNA was detected

Figure 3 Effect of IGF-I, ACTH, GHRH and DEX treatment on GH gene in mouse pituitary cells in primary culture. (A) Autoradiogram of the results of a Northern blotting analysis of RNA prepared from pituitary cells treated with IGF-I, ACTH, GHRH and DEX for 24 h. The upper band indicates GH mRNA, and the lower band indicates β-actin mRNA. (B) Summary of Northern blotting analysis of GH mRNA levels. The values represent the relative level of GH mRNA compared with the level in control cells without GH treatment which was defined as 1.0. In each sample, GH mRNA levels were normalized relative to the amount of β-actin mRNA. Each value is the mean $\pm$ S.E.M. of three different preparations of RNA. * $P < 0.05$ compared with control.

(Fig. 8C and D, K and L). GH receptor mRNA was not detected in corticotrophs and gonadotrophs (Fig. 8E and F, G and H, I and J).

Discussion

Pituitary IGF-I exerts local actions to regulate pituitary functions within the gland, since IGF-I-binding sites (Goodyer *et al.* 1984b) and the mRNAs encoding the IGF-I receptor and binding proteins are found in the pituitary (Bach & Bondy 1992). We have previously found expression of IGF-I receptor mRNA in mouse somatotrophs (Honda *et al.* 1998), and have shown the mitogenic action of IGF-I on corticotrophs and mammotrophs (Oomizu *et al.* 1998). In the present study, we have demonstrated that IGF-I suppressed GH synthesis and stimulated POMC synthesis at the transcription level. IGF-I treatment significantly decreased GH mRNA levels. These results are consistent with previous reports

Figure 4 Effect of IGF-I treatment on POMC gene expression in mouse pituitary cells in primary culture. (A) Autoradiogram of the results of Northern blotting analysis of RNA prepared from pituitary cells treated with IGF-I for 24 h. The upper band indicates POMC mRNA, and the lower band indicates β-actin mRNA. (B) Summary of Northern blotting analysis of POMC mRNA levels. The values represent the relative level of POMC mRNA compared with the level in control cells without IGF-I treatment which was defined as 1.0. In each sample, POMC mRNA levels were normalized relative to the amount of β-actin mRNA. Each value is the mean ± S.E.M. of three different preparations of RNA. * $P < 0.05$ compared with control.

that IGF-I attenuates GH expression and secretion at the pituitary level (Goodyer *et al.* 1984a, Yamashita & Melmed 1986, Ceda *et al.* 1987, Yamasaki *et al.* 1991). In addition, pituitary IGF-I gene transcription was enhanced by GH treatment, which was clarified using the competitive RT-PCR method. This is the first report showing direct evidence of GH-induced IGF-I synthesis in mouse pituitary cells as far as we know. Together, these results lead us to the hypothesis that pituitary IGF-I is an inhibitory mediator of GH for the regulation of GH gene expression, and a stimulatory mediator for POMC gene expression. It is probable that GH first stimulates IGF-I expression in somatotrophs in an autocrine manner and then, in turn, the elevated IGF-I level within the pituitary gland diminishes GH synthesis.

Figure 5 Effect of GH treatment on POMC gene expression in mouse pituitary cells in primary culture. (A) Autoradiogram of the results of a Northern blotting analysis of RNA prepared from pituitary cells treated with GH for 4 days. The upper band indicates GH mRNA, and the lower band indicates β-actin mRNA. (B) Summary of Northern blotting analysis of POMC mRNA levels. The values represent the relative level of POMC mRNA compared with the level in control cells without GH treatment which was defined as 1.0. In each sample, POMC mRNA levels were normalized relative to the amount of β-actin mRNA. Each value is the mean ± S.E.M. of three different preparations of RNA. * $P < 0.05$ compared with control.

Figure 6 Northern blotting analysis of GH receptor mRNA in the mouse. Total RNA (20 μg) derived from pituitaries was used. Mouse GH receptor cDNA probe was used. 4.2 kb GH receptor mRNA and 1.4 kb GHBP mRNA were detected.

IGF-I is synthesized predominantly in the liver under the control of GH (Schwander *et al.* 1983, Lowe *et al.* 1987, Shoba *et al.* 2001), and acts as a mediator of the

Figure 7 *In situ* hybridization analysis of GH receptor mRNA in mouse pituitary glands. (A) GH receptor mRNA-expressing cells (arrow) were detected in anterior lobe (a), but in the intermediate lobe (i) or posterior lobe (p), GH receptor mRNA-expressing cells were not detected. Bar=50 μ m. (B) GH receptor mRNA-expressing cells in anterior pituitary cells (arrow). Bar=30 μ m. (C) *In situ* hybridization using pBR328 as probe. No hybridization signals were detected. Bar=30 μ m.

growth-promoting actions of GH (LeRoith *et al.* 1995). The IGF-I gene is expressed in multiple extrahepatic tissues, including the pituitary gland (D'Ercole *et al.* 1984). Possible autocrine and paracrine actions of IGF-I have been recognized in the pituitary gland (Yamasaki *et al.* 1991, Bach & Bondy 1992, Honda *et al.* 1998, Oomizu *et al.* 1998). We have investigated whether or not GH affected pituitary IGF-I synthesis in a similar manner to the hepatic IGF-I synthesis in mice. Treatment of mouse pituitary cells with GH at concentrations of 0.5 and 1 μ g/ml, which were chosen according to previous data on serum GH levels in mice (Sinha *et al.* 1977), were carried out, and we found that GH treatment increased IGF-I mRNA levels. Since somatotrophs are the source of pituitary IGF-I (Honda *et al.* 1998) and the expressed GH

receptor mRNA, and probably the GH-receptor protein, as evidenced by the presence of GH-receptor mRNA transcripts in the present study, we have concluded that GH directly stimulates IGF-I gene expression through the GH receptor expressed on somatotrophs.

GHRH treatment significantly increased GH expression as expected, but did not have any significant effect on IGF-I synthesis, which is in agreement with a previous report that chronic administration of a potent agonist of GHRH did not change serum IGF-I levels (Kovács *et al.* 1996). These results indicate that pituitary IGF-I synthesis is not directly regulated by hypothalamic GHRH.

Estrogen is known to be a stimulator of IGF-I expression in the pituitaries of ovariectomized and estrous rats (Michels *et al.* 1993). However, E2 treatment failed to stimulate IGF-I expression in the mouse pituitary. These discrepancies may be due to differences in the animal species and/or the sex used, and/or the experimental schedules. In our *in vitro* study, the steroid treatment was continued for only 24 h, whereas Michels *et al.* (1993), for example, implanted pellets containing E2 for 54 days *in vivo*. Up-regulation of IGF-I transcription in the pituitary glands probably requires such chronic treatment with E2.

Several previous reports have shown that exogenous GH treatment caused suppression of the endogenous pulsatile GH secretion in rats (Tannenbaum 1980, Willoughby *et al.* 1980). Considering these previous findings, we studied the effect of GH on GH synthesis. GH treatment did not change the GH mRNA levels. A similar result for cultured rat anterior pituitary cells showing that GH treatment (200 and 1000 ng/ml) for 1 day did not inhibit GH release has been reported (Goodyer *et al.* 1984a). These results suggest that GH expression is not regulated by the direct action of GH in mice, and this is in agreement with previous studies performed in rats that GH autoregulates its own secretion in the hypothalamus, but not in the pituitary (Richman *et al.* 1981, Kraicer *et al.* 1988).

IGF-I is synthesized in various areas of the brain, including the hypothalamus (García-Segura *et al.* 1991, Dueñas *et al.* 1994), and IGF-I receptors are also present in the brain (Werther *et al.* 1990, Kar *et al.* 1993). Several reports have indicated that IGF-I also takes part in the regulation of GH secretion in the hypothalamus (Tannenbaum *et al.* 1983, Yamashita & Melmed 1986). However, it is not clear whether circulating IGF-I or IGF-I synthesized in the brain affects GHRH and somatostatin release from the hypothalamus. From the findings in the present study, we have concluded that IGF-I regulates GH synthesis at the pituitary level as well as at the hypothalamic level.

The expression of POMC mRNA was stimulated by IGF-I treatment. However, Goodyer *et al.* (1984a) reported that the release of ACTH, one of the POMC-derived peptides, remained unaltered with IGF-I treatment. This

Figure 8 Analysis of cell types of GH receptor mRNA-expressing cells. *In situ* hybridization detection of GH receptor mRNA (A, C, E, G, I and K) and immunocytochemical detection of GH (B), PRL (D), ACTH (F), TSH β (H), LH β (J) and FSH β (L) were simultaneously carried out. A and B, C and D, E and F, G and H, I and J, and K and L are the same sections respectively. A GH-immunoreactive cell (B, arrow) expressed GH receptor mRNA (A, arrow). A PRL-immunoreactive cell (D, arrow) and an FSH β -immunoreactive cell (L, arrow) expressed GH receptor mRNA (C and K, arrows). However, other PRL-immunoreactive cells (D, arrowhead) and FSH β -immunoreactive cells (L, arrowhead) did not express GH receptor mRNA (C and K, arrowheads). ACTH (E and F)-, TSH β (G and H)- and LH β (I and J)-immunoreactive cells did not express GH receptor mRNA (arrowheads). Bar=10 μ m.

discrepancy remains to be clarified, but may be ascribed to post-transcriptional inhibition of POMC expression by the accumulation of POMC mRNA. As some corticotrophs express IGF-I receptor mRNA (Honda *et al.* 1998), the present findings lead us to the conclusion that pituitary IGF-I acts directly on the corticotrophs, and stimulates POMC gene expression in a paracrine manner in the mouse pituitary gland. POMC gene expression in

corticotrophs appears to be regulated co-ordinately by two factors, hypothalamic corticotropin-releasing hormone and pituitary IGF-I.

A 4-day exposure to rat GH (4 and 8 μ g/ml) significantly increased the POMC mRNA levels. We estimated that these concentrations of GH correspond to approximately 10^{-7} M, although we had no information about the purity of the GH preparation used. The stimulatory

effect of GH on POMC mRNA production appears to be indirect, since GH receptor mRNA signals were not detected in corticotrophs. Therefore, it is probable that GH-induced POMC mRNA production is mediated by factors such as IGF-I whose synthesis is stimulated by GH.

The hypothalamo–corticotroph–adrenal axis has been extensively investigated with respect to the stress response, and energy and electrolyte metabolism. Activation of the hypothalamo–corticotroph–adrenal axis partially antagonizes the somatic growth regulated by the hypothalamo–somatotroph–IGF-I axis. Glucocorticoids, e.g. DEX, are potent inhibitors of linear growth and GH secretion when secreted or administered in pharmacological amounts *in vivo* (Luo & Murphy 1989, Ohyama *et al.* 1997). DEX inhibited GHRH expression in the hypothalamus, but increased GH expression (Evans *et al.* 1982, Lam & Srivastava 1997). Treatment of mouse pituitary cells with DEX decreased IGF-I mRNA levels, and this is consistent with previous reports showing that DEX exerted potent inhibitory effects on IGF-I production in many types of cells (Luo & Murphy 1989, Delany & Canalis 1995). This seems to represent the negative feedback loop of glucocorticoid secretion, since pituitary IGF-I stimulates POMC gene expression in a paracrine manner, probably resulting in the enhanced production of ACTH. These findings provide the hypothesis that somatotrophs directly regulate corticotroph functions with IGF-I molecules, and that corticotrophs indirectly regulate somatotrophs being mediated by glucocorticoids. The somatotroph–corticotroph interaction at the pituitary level is implicated in the co-ordination of the stress response and regulation of somatic growth.

The present study has demonstrated the expression of GH receptor mRNA in the mouse anterior pituitary with RT-PCR. Northern blotting analysis using the mouse GH receptor cDNA probe (pmghr1-1) demonstrated two transcripts, the 4.2 kb GH receptor mRNA, and the 1.4 kb GHBP mRNA. This indicates that the mouse GH receptor cDNA probe used in the present study bound to both GH receptor mRNA and GHBP mRNA. Therefore, the GH receptor cDNA probe cannot differentiate GH receptor mRNA from GHBP mRNA. GH receptor mRNA and protein were detected in somatotrophs and non-GH-secreting cells (Fraser *et al.* 1991). These results are in agreement with the present finding that both GH receptor mRNA and GHBP mRNA were detected in mouse anterior pituitary glands, particularly in somatotrophs and some of the mammatrophs and gonadotrophs, while Ilkbar *et al.* (1995) failed to find GH receptor mRNA expression in the mouse pituitary. The reason for this discrepancy is not clear. However, the *in vitro* physiological evidence of GH-induced IGF-I synthesis strongly indicates the expression of GH receptor in somatotrophs. GHBP within the cytoplasm of the anterior pituitary cells may have a role in modulating GH metabolism and GH action (Herington *et al.* 1991). GHBP stabilizes stored GH

within somatotrophs and is co-secreted with GH molecules into the blood. Thus, GHBP regulates the number of GH molecules around the receptor site. From these observations, it is very likely that GH receptor and GHBP synthesized in pituitary glands regulate the functions of somatotrophs in an autocrine and/or paracrine manner.

Many lines of evidence have shown that a cell-to-cell interaction between pituitary secretory cells is important in the regulation of the proliferation and functions of pituitary cells, and that this interaction is mediated by hormones, growth factors and cytokines synthesized in the pituitary cells (Renner *et al.* 1996, Ray & Melmed 1997, Schwartz 2000). In the present study, we have demonstrated that GH stimulated IGF-I synthesis in somatotrophs, and that IGF-I inhibited GH expression and stimulated POMC expression in corticotrophs. These findings suggest the presence of autocrine and paracrine actions of somatotrophic IGF-I within the pituitary gland, and a possible interaction between somatotrophs and corticotrophs.

Acknowledgements

The authors would like to thank the National Hormone and Pituitary Program, NIDDK, NIH, Bethesda, MD, USA for the kind supply of antibodies to pituitary hormones and purified pituitary hormones, and Dr J A Martial for generously supplying the rat GH cDNA probe. This study was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science to S T.

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Received 1 April 2003

Accepted 4 April 2003