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Efficacy and safety of growth hormone (GH) in the treatment of adult Japanese patients with GH deficiency: A randomised, placebo-controlled study ☆

K. Chihara [a,*], Y. Kato [b], H. Kohno [c], K. Takano [d], T. Tanaka [e], A. Teramoto [f], A. Shimatsu [g]

[a] *Division of Endocrinology, Metabolism, Hematology and Oncology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-2 Kusunoki-cho Chuo-ku, Kobe 650-0017, Japan*
[b] *Division of Endocrinology, Metabolism, Hematology and Oncology, Shimane University, Izumo, Japan*
[c] *Department of Endocrinology and Metabolism, Fukuoka Children's Hospital, Fukuoka, Japan*
[d] *Department of Internal Medicine 2, Tokyo Women's Medical University School of Medicine, Tokyo, Japan*
[e] *Division of Endocrinology and Metabolism, National Center for Child Health and Development, Tokyo, Japan*
[f] *Department of Neurosurgery, Nippon Medicine School, Tokyo, Japan*
[g] *Clinical Research Center for Endocrine and Metabolic Disease, Kyoto Medical Center, Kyoto, Japan*

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Abstract

Objective: The aim of this study was to assess the effect of growth hormone (GH) replacement therapy on lean body mass (LBM) and other variables including body fat mass, serum lipids and quality of life measures in GH-deficient Japanese adults.
Design: This was a multicentre, double-blind, placebo-controlled, parallel group study. Following initial screening, patients were randomly assigned to GH treatment ($n = 37$) or placebo ($n = 36$). GH treatment was started at an initial dose 0.003 mg/kg/day s.c. each day for the first 4 weeks after which the dose was increased to 0.006 mg/kg/day for 4 weeks and then to 0.012 mg/kg/day for the last 16 weeks ($n = 37$). Body composition, serum lipids, serum IGF-I and IGFBP-3 levels were measured during the 24-week study. Short Form-36 and Quality of Life Assessment of GH Deficiency in Adults scores were also determined.
Results: LBM was significantly increased from baseline at 24 weeks in GH-treated patients, with a mean (±SD) increase of 4.7% (±5.3%) compared with an increase of 1.0% (±4.4%) in the placebo group ($p < 0.0001$ versus baseline, $p = 0.0003$ versus placebo). Percentage body fat decreased significantly from baseline in GH-treated patients (9.3%, $p < 0.0001$), compared with a non-significant 0.2% increase in the placebo group ($p < 0.0004$ for difference between treatment groups). In addition, significantly increased serum IGF-I and IGFBP-3 levels and improvements in the patients' serum lipid profiles were observed in patients who received GH therapy. Changes in quality of life measures did not differ between treatments, probably because of the small number of patients studied. GH therapy was well tolerated, with adverse events of any cause reported in 86.5% of the GH treatment group and 83.3% of the placebo group.
Conclusion: GH treatment significantly improved body composition and serum lipid profiles in adult Japanese patients with GH deficiency compared with placebo and had no clinically relevant adverse effects.

Keywords: Growth hormone treatment; Growth hormone deficiency; Japanese adults; Body composition

☆ A multicentre study in Japan coordinated by the Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan.

* Corresponding author. Tel.: +81 78 382 5885; fax: +81 78 382 5899.
E-mail address: chiharak@med.kobe-u.ac.jp (K. Chihara).

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1. Introduction

While the deleterious effects of GH deficiency (GHD) and the benefits of GH replacement therapy in Caucasian patients are well established [1–3], there are fewer data concerning Japanese or other Oriental populations. Epidemiological surveys have indicated that in addition to approximately 29,000 short stature children registered for GH therapy in Japan, there are about 7000 adult patients with hypopituitarism [4,5]. GHD has important clinical consequences, as demonstrated by Irie et al. in a study of 507 Japanese adults with GHD, which showed that these subjects had a higher prevalence of hypertension, hyperlipidaemia, myocardial infarction and angina pectoris compared with 362 patients with non-GHD hypopituitarism [6]. These factors together constitute a considerable increase in cardiovascular risk. Although no significant difference in overall mortality or cause of death was detected between the two groups by Irie and colleagues, it is notable that on average the GHD patients died 10 years earlier than the non-GHD group [6].

There is no clear evidence that the GH-IGF-I system differs between Caucasians and Orientals, even though the risks of obesity and cardiovascular events may be different in the two ethnic groups. It is widely thought that the response to GH replacement therapy in adult Oriental patients with GHD should be similar to the response in Caucasians, in terms of the effects on body composition and serum lipid parameters. Recently, an analysis of a nationwide autopsy database by Kaji and Chihara found that cerebrovascular disease was higher in Japanese patients with hypopituitarism than in an age- and gender-matched control population [7]. Another study in Japanese patients with GHD of either childhood onset (CO) or adult onset (AO) [8] has shown that, as in Caucasian adults with GHD [9], these subjects had an increased intima–media thickness of the carotid artery, another known cardiovascular risk factor.

Together these data indicate that GHD in Japanese adults represents an important healthcare concern with similar significant adverse effects on health outcomes as observed in Caucasian subjects. Effective treatment for these patients is thus required. Encouraging results have been recently reported for treatment of 64 Japanese adult GHD patients with GH replacement therapy [10], with a significant increase in lean body mass (LBM) and a decrease in body fat mass (BFM) observed. The present study adds to the currently small body of data concerning treatment of Japanese adult GHD patients with GH replacement therapy. The effects of six months of GH replacement therapy with recombinant human GH (Genotropin®, Pfizer) on body composition, serum lipids and quality of life (QoL) measures were assessed in 73 Japanese adults with GHD of either CO or AO.

2. Materials and methods

This randomised, double-blind, placebo-controlled study was conducted at 21 clinical centres in Japan. The study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki (1964 and subsequent revisions), and written informed consent was obtained from all patients entering the study. The primary aim of the study was to assess the effects of GH treatment on patients' LBM. The effects of treatment on BFM, patients' serum lipid profiles, serum insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3), symptoms, QoL, and safety were also determined as secondary endpoints.

Japanese adults aged 18–65 years with organic or idiopathic, isolated or multiple, CO or AO GHD of at least 2 years' duration were recruited into the study. CO patients were defined as having onset of GHD before age 18 years and AO patients as having onset of GHD at age 18 years or later. A diagnosis of GHD was made if a patient had a peak GH response of less than 3.0 ng/mL in a GH stimulation test (usually insulin-induced hypoglycaemia but arginine or glucagon were used if hypoglycaemia was contraindicated). Patients with multiple pituitary hormone deficiency had to have been on stable replacement therapy for at least 6 months prior to study entry. For those patients for whom there was an organic cause of GHD, at least 2 years had to have elapsed since completion of surgery or radiotherapy, and there had to be no evidence of exacerbation of the primary disease on computerised tomography (CT) or magnetic resonance imaging (MRI) within the year prior to study entry or within 6 months of the study screening tests conducted before patient enrolment.

Patients were excluded from the study if they had received GH treatment within the past year or if they had experienced any serious illness within the last 6 months. Patients were also excluded if they had serious cardiac, hepatic or renal disease, uncontrolled hypertension, diabetes mellitus, acromegaly, or any malignant tumour. Female patients were excluded if they were known or suspected to be pregnant or lactating. Other exclusion criteria included long-term medication with corticosteroids at a dosage used in pharmacological therapy or with anabolic steroids for therapy other than gonadal steroid replacement, or contraceptive treatment during the 3 months before providing informed consent to study participation. Patients receiving replacement therapy for other pituitary hormone deficiencies were not excluded, but should have been on the appropriate replacement therapy for at least 6 months, with temporary dose adjustment if necessary. For patients receiving sex hormone substitution, the dosage of replacement therapy was not varied during the study period.

2.1. Study treatment

Patients were randomised to 24 weeks of double-blind treatment with either recombinant human GH (Genotropin®, Pfizer) or matching placebo. Patients in the active treatment group received an initial dose of GH of 0.021 mg/kg/week, administered by subcutaneous (s.c.) injection of 0.003 mg/kg each day before bedtime for the first 4 weeks. The dose of GH was increased to 0.042 mg/kg/week for the second 4 weeks of treatment and then raised again to 0.084 mg/kg/week for the remaining 16 weeks of the study period. The patients in the placebo group received injections of placebo each night throughout the study. The study medications were self-administered by the patients using cartridge pens, and placebo was matched by administering an excipient solution using the same number of clicks of the pen to maintain treatment blinding. Dose reductions of 25–50% were permitted at the investigators' discretion in the event of GH-induced side effects.

All patients attended study clinics for assessment at an initial screening visit, at the time of starting GH replacement therapy and at 4, 8, 12 weeks and 24 weeks. Body composition (LBM and BFM) was assessed using dual energy X-ray absorptiometry (DEXA) at baseline at the time of starting GH therapy, and at 12 and 24 weeks. All DEXA measurements performed at each of the participating clinical centres were blinded and evaluated centrally at the Department of Radiology, Kawasaki Medical School, Kurashiki City, Okayama, Japan. Quality control (QC) of DEXA equipment at each clinical centre was performed using both synthetic tissue of defined standard (a calibration phantom) at least once a week and a whole body phantom every 6 months. The QC data were also blinded and evaluated centrally at the same school. Serum levels of IGF-I and IGFBP-3 were determined using standard immunoradiometric assays, while serum lipids (total, high-density lipoprotein [HDL]- and low-density lipoprotein [LDL]-cholesterol, triglycerides, phosphatide, non-esterified fatty acids [NEFA]) were determined using enzymatic methods. Biochemical samples were analysed centrally (MBC Inc, Itabashi-ku, Tokyo, Japan).

Subjective and objective symptoms were assessed at each clinic visit from screening. Parameters of QoL were assessed by the Japanese version of the Short Form-36 (SF-36) and a Japanese translation of the Quality of Life Assessment of GH Deficiency in Adults (QoL-AGHDA) at baseline, 12 weeks and 24 weeks. For each assessment of QoL, a patient completed a QoL questionnaire, which was then sealed in an envelope for later analysis; investigators did not assess QoL directly. Safety was assessed by recording all adverse events reported by patients or investigating physicians during the study period, which were coded according to the World Health Organization Adverse Reaction Terminology. Laboratory measurements of thyroid hormones, haemoglobin A_{1c} concentrations, total protein, electrolytes, and liver and kidney function were performed centrally. Anti-GH antibodies were measured centrally at the start of therapy and at 24 weeks.

2.2. Statistics

The primary efficacy endpoint, the percent change from baseline in patients' LBM after 24 weeks of GH replacement therapy or placebo, was compared between the treatment groups using the two-sided Wilcoxon rank sum test. Comparisons between baseline and week 24 values within each treatment group were analysed using the two-sided Wilcoxon signed rank test. Secondary endpoints were analysed in the same way as the primary endpoint. All statistical tests used a significance level of 5%. SD scores were calculated for serum IGF-I and IGFBP-3 values by comparison with age- and gender-matched Japanese subjects. With data distributions resembling a normal distribution, results for LBM and BFM are presented as summary statistics (mean and SD). For other endpoints, median minimum and maximum values are also presented.

The primary efficacy analysis used the intent-to-treat (ITT) population, which comprised all patients who received at least one dose of study treatment and for whom at least one post-treatment assessment was performed. Study endpoints were also assessed for the per protocol (PP) population. Safety analyses were performed on the ITT population.

3. Results

Of 90 subjects screened for study entry, 75 patients were randomised to treatment. One patient subsequently withdrew consent and another was discontinued early from the study due to loss of follow-up, so that the ITT population comprised 73 patients of whom 37 patients received GH and 36 patients placebo. The PP population comprised 34 patients in each treatment group, after excluding 3 patients in the GH group and 2 patients in the placebo group with major protocol violations.

In the GH treatment group, 2 patients discontinued because of adverse events and 1 patient was excluded because their dose of gonadal steroid replacement changed during study period. In the placebo group, 1 patient was poorly compliant with study treatment (administered doses less than 50% of assigned doses), and a second patient received additional gonadal steroid replacement at the time of starting GH treatment.

The baseline demographic characteristics of the patients are summarised in Table 1. There were no

Table 1
Baseline characteristics of Japanese adult GHD patients by assigned treatment group (GH or placebo) and according to time of onset of GHD (adult-onset, AO, or childhood-onset, CO)

	GH			Placebo		
	All ($n = 37$)	CO ($n = 19$)	AO ($n = 18$)	All ($n = 36$)	CO ($n = 19$)	AO ($n = 17$)
Male/female	23/14	12/7	11/7	23/13	13/6	10/7
Age	37.6 ± 13.0	29.2 ± 4.7	46.5 ± 13.2	36.7 ± 12.9	29.3 ± 5.9	45.0 ± 13.7
Height (cm)	163.0 ± 10.1	165.0 ± 9.5	160.8 ± 10.4	161.6 ± 9.9	162.5 ± 7.9	160.5 ± 11.9
Weight (kg)	66.9 ± 13.6	66.2 ± 11.8	67.7 ± 15.6	62.8 ± 14.6	62.6 ± 13.8	63.1 ± 15.9
BMI (kg/m^2)	25.1 ± 3.8	24.2 ± 3.4	25.9 ± 4.2	23.9 ± 4.4	23.7 ± 4.6	24.2 ± 4.1
LBM (kg)	41.5 ± 9.8	40.3 ± 8.4	42.8 ± 11.1	39.9 ± 10.3	38.1 ± 8.5	42.0 ± 12.0
BFM (kg)	22.1 ± 7.2	22.3 ± 7.7	22.0 ± 6.7	19.9 ± 7.3	20.8 ± 8.6	18.9 ± 5.7
IGF-I (μg/L)	77.1 ± 60.2	46.6 ± 35.6	109.4 ± 64.7	83.3 ± 50.5	69.0 ± 36.2	99.4 ± 59.9
IGF-I SD score	−2.0 ± 1.6	−3.1 ± 0.9	−0.8 ± 1.4	−1.8 ± 1.3	−2.4 ± 1.0	−1.1 ± 1.3
IGFBP-3 (mg/L)	2.0 ± 1.1	1.5 ± 1.0	2.5 ± 1.0	2.0 ± 0.8	1.8 ± 0.6	2.3 ± 1.0
IGFBP-3 SD score	−3.8 ± 4.0	−5.9 ± 4.1	−1.6 ± 2.6	−3.2 ± 2.8	−3.9 ± 2.6	−2.3 ± 2.7

Data shown as mean ± SD for the ITT population.
BMI, body mass index; LBM, lean body mass; BFM, body fat mass; IGF-I, insulin-like growth factor; IGFBP-3, IGF binding protein-3.

significant differences in demographic parameters between the two treatment groups. Both groups comprised approximately two-thirds male patients and each had a mean age of about 37 years.

The majority of patients (~95%) in both treatment groups had multiple pituitary hormone deficiency; only 2 patients in each group had isolated GHD. With regard to aetiology, the major causes of hypopituitarism in both groups were tumours, which occurred in 25 patients in the GH treatment group and in 27 of the placebo group. The most common tumour types were germinoma (reflecting the known relatively high prevalence of this tumour type in Japan [11]), then craniopharyngioma and pituitary adenomas. Six patients (GH treatment group, 16.2%; placebo group, 16.7%) in each treatment group had idiopathic hypopituitarism. The treatment groups were generally well matched with regard to patients' baseline height, weight and body composition profiles. Any differences between the baseline characteristics of the two groups were slight and not significant.

In total, 54.1% of patients in the GH treatment group and 47.2% of those in the placebo group had previously received GH replacement therapy. With regard to other hormone replacement therapy, the only significant difference between groups was in the proportion of patients who had received gonadotropin treatment (83.3% of those in the GH treatment group compared with 58.3% of those in the placebo group ($p < 0.05$). For almost all patients receiving this replacement therapy, the dosage of gonadotropin was not changed during study period.

About half of the patients in each treatment group had CO GHD (Table 1). Mean (±SD) values of IGF-I SD score at the start of treatment were below the normal range standardised for age and gender in patients with CO GHD, whereas values in those with AO GHD were in the lower level of the reference range.

3.1. Efficacy

3.1.1. Body composition

There was a significant increase in LBM from baseline to week 24 in the GH treatment group compared with the placebo-treated patients ($p = 0.0003$; Fig. 1, Table 2). Mean LBM increased by 4.7%, from 41.5 kg at baseline to 43.4 kg at week 24, in the patients who received active GH treatment, whereas in the placebo group there was a non-significant 1.0% increase from 39.9 kg at baseline to 40.4 kg at week 24 (Table 2).

The beneficial effect of GH treatment on LBM was apparent as early as week 12 of the study, at which point mean LBM had increased to 43.2 ± 9.7 kg in the GH treatment group (4.6 ± 4.7% increase). In the placebo group, an increase in LBM of 0.6 ± 4.4% to 40.3 kg was recorded at this time point.

Mean BFM was significantly reduced from baseline after 24 weeks of GH treatment but not with placebo (Table 2). In the GH treatment group mean (SD)

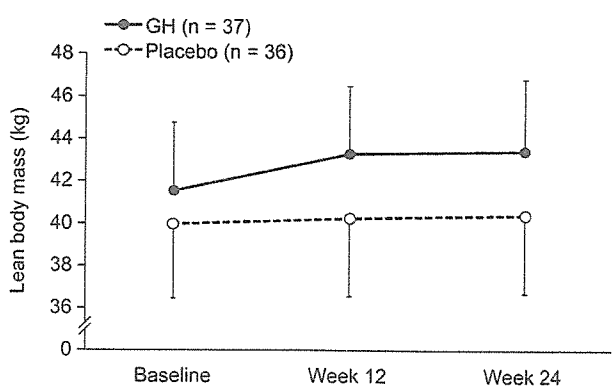

Fig. 1. Changes from baseline in mean lean body mass (LBM) in patients who received GH treatment or placebo (ITT population). Data shown as mean and, for clarity, upper 95% CI (GH) and lower 95% CI (placebo).

Table 2
Mean percent changes in lean body mass (LBM) and body fat mass (BFM) at 24 weeks in Japanese adults treated with GH replacement therapy or placebo (ITT population)

	GH ($n = 37$)		Placebo ($n = 36$)		GH vs placebo[b]
	Baseline	% Change at week 24[a]	Baseline	% Change at week 24[a]	
LBM					
Mean ± SD	41.5 ± 9.8	4.7 ± 5.3*	39.9 ± 10.3	1.0 ± 4.4	$p = 0.0003$
95% CI	[38.2, 44.7]	[3.0, 6.5]	[36.4, 43.4]	[−0.5, 2.5]	
BFM					
Mean ± SD	22.1 ± 7.2	−9.3 ± 11.8*	19.9 ± 7.3	0.2 ± 7.1	$p = 0.0004$
95% CI	[19.8, 24.5]	[−13.2, −5.4]	[17.4, 22.4]	[−2.2, 2.6]	

[a] Wilcoxon signed rank test (*$p < 0.05$).
[b] Wilcoxon rank sum test.

BFM was significantly reduced by 9.3%, from 22.1 ± 7.2 kg at baseline to 20.2 ± 7.5 kg at week 24. In the placebo group a non-significant 0.2% increase in mean BFM, from 19.9 ± 7.3 kg at baseline to 19.9 ± 7.3 kg at week 24, was observed. The change in BFM in the GH treatment group was significant compared with placebo ($p = 0.0004$). There was no significant change in patients' mean weight from baseline to week 24 in either treatment group.

3.1.2. Serum IGF-I and IGFBP-3

In the GH treatment group mean serum IGF-I levels at week 24 were significantly increased from baseline (Table 3). In contrast, in the placebo group there was a non-significant increase compared with baseline at week 24. The average change in IGF-I levels after 24 weeks' GH treatment was 2.8 ± 1.6, which resulted in a normalisation of the mean IGF-I SD score (from −2.0 ± 1.6 to 0.8 ± 2.4). The change in IGF-I SD scores at week 24 in the GH treatment group was significantly greater than that in the placebo group ($p < 0.0001$).

Mean serum IGFBP-3 levels were also significantly increased from baseline at 24 weeks in the GH treatment group but not in the placebo group (Table 3). Similarly, the change in IGFBP-3 SD scores at week 24 in the GH-treated patients was statistically significant compared with baseline and with the change observed in placebo-treated patients ($p < 0.0001$).

3.1.3. Serum lipids

The changes in patients' serum lipid profiles are summarised in Table 4. In the GH treatment group, there were significant changes in serum levels of total cholesterol, LDL-cholesterol and NEFA at week 24 compared with baseline. In comparison, there were no significant changes in any serum lipids at week 24 compared with

Table 3
Changes in serum IGF-I and IGFBP-3 levels from baseline to week 24 in Japanese adults treated with GH replacement therapy or placebo (ITT population)

	GH ($n = 37$)		Placebo ($n = 36$)		GH vs placebo[b]
	Baseline	Change at week 24[a]	Baseline	Change at week 24[a]	
IGF-I (μg/L)					
Mean ± SD	77.1 ± 60.2	161.9 ± 113.3*	83.3 ± 50.5	4.2 ± 22.2	$p < 0.0001$
Median	68.1	152.5	77.2	2.3	
[Min, max]	[10.5, 253.0]	[−35.9, 404.0]	[10.3, 196.0]	[−44.0, 73.0]	
IGF-I SD score					
Mean ± SD	−2.0 ± 1.6	2.8 ± 1.6*	−1.8 ± 1.3	0.1 ± 0.4	$p < 0.0001$
Median	−2.3	3.0	−2.1	0.1	
[Min, max]	[−4.2, 1.0]	[−0.9, 5.5]	[−4.3, 0.9]	[−0.8, 1.3]	
IGFBP-3 (mg/L)					
Mean ± SD	2.0 ± 1.1	1.0 ± 0.8*	2.0 ± 0.8	0.1 ± 0.4	$p < 0.0001$
Median	2.1	1.1	2.0	0.1	
[Min, max]	[0.4, 4.7]	[−1.8, 2.2]	[0.5, 3.8]	[−0.5, 1.6]	
IGFBP-3 SD score					
Mean ± SD	−3.8 ± 4.0	2.9 ± 2.9*	−3.2 ± 2.8	0.4 ± 1.3	$p < 0.0001$
Median	−2.8	2.6	−2.4	0.4	
[Min, max]	[−13.5, 2.4]	[−3.9, 9.8]	[−10.9, 1.1]	[−1.5, 4.2]	

[a] Wilcoxon signed rank test (*$p < 0.05$).
[b] Wilcoxon rank sum test.

Table 4
Changes in serum lipid profiles from baseline to week 24 in Japanese adults treated with GH replacement therapy or placebo (ITT population)

	GH ($n = 37$)		Placebo ($n = 36$)		GH vs placebo[c]
	Baseline	Change at week 24[b]	Baseline	Change at week 24[b]	
Total cholesterol (mmol/L)[a]					
Mean ± SD	5.32 ± 1.13	−0.30 ± 0.56*	5.60 ± 0.90	0.06 ± 0.62	$p = 0.0390$
Median	4.99	−0.26	5.66	−0.01	
[Min, max]	[3.44, 9.00]	[−1.55, 0.93]	[3.23, 8.12]	[−1.14, 1.40]	
HDL-cholesterol (mmol/L)[a]					
Mean ± SD	1.35 ± 0.39	0.07 ± 0.23	1.28 ± 0.34	0.02 ± 0.22	$p = 0.4009$
Median	1.27	0.08	1.27	0.04	
[Min, max]	[0.62, 2.53]	[−0.34, 0.75]	[0.75, 2.22]	[−0.39, 0.65]	
LDL-cholesterol (mmol/L)[a]					
Mean ± SD	3.30 ± 0.98	−0.36 ± 0.51*	3.64 ± 0.77	−0.11 ± 0.60	$p = 0.0645$
Median	3.21	−0.41	3.66	−0.09	
[Min, max]	[1.27, 6.23]	[−1.40, 0.62]	[2.22, 6.21]	[−1.50, 0.91]	
Triglyceride (mmol/L)[a]					
Mean ± SD	2.13 ± 1.82	−0.33 ± 1.40	2.07 ± 1.19	0.00 ± 1.18	$p = 0.4732$
Median	1.49	−0.20	1.98	−0.07	
[Min, max]	[0.51, 9.45]	[−5.92, 2.22]	[0.37, 4.86]	[−1.97, 3.13]	
NEFA (mEq/L)					
Mean ± SD	0.36 ± 0.21	0.10 ± 0.26*	0.41 ± 0.21	0.03 ± 0.27	$p = 0.2063$
Median	0.33	0.10	0.41	0.05	
[Min, max]	[0.08, 0.82]	[−0.42, 0.73]	[0.08, 0.91]	[−0.39, 0.85]	
Phospholipids (mmol/L)[a]					
Mean ± SD	3.12 ± 0.75	−0.14 ± 0.49	3.14 ± 0.43	0.00 ± 0.44	$p = 0.3021$
Median	3.03	−0.13	3.17	−0.01	
[Min, max]	[2.05, 5.33]	[−1.51, 0.84]	[1.92, 4.00]	[−0.76, 0.95]	

NEFA, non-esterified fatty acids.
[a] Calculated using respective molecular weights.
[b] Wilcoxon signed rank test (*$p < 0.05$).
[c] Wilcoxon rank sum test.

baseline in the placebo group. At week 24, mean total cholesterol had significantly decreased by 0.30 mmol/L in the GH treatment group and increased slightly in the placebo group. The difference between the treatment groups for the change in mean total cholesterol at 24 weeks was statistically significant ($p = 0.039$); differences between the two groups in other lipid changes were not significant (Table 4).

3.1.4. Symptom scores

All symptom scores were reduced from baseline values in both treatment groups. Decreased motor ability and/or muscle strength was the most frequently observed symptom at baseline. The improvements in symptoms appeared to be greater in the GH treatment group, although no formal statistical analysis was performed (Fig. 2).

3.1.5. Quality of life (QoL) parameters

Most QoL parameters of the SF-36 assessment were improved from baseline at week 24 in the GH treatment group. In the placebo group improvements were also recorded in the scores for awareness of general health, vitality and mental health, which were reduced at baseline; however, there were no significant differences between GH treatment group and placebo group for any parameter. The change in mean QoL-AGHDA score was small in both the GH treatment group and the placebo group and there was no significant difference between the two groups in QoL-AGHDA score ($p = 0.5588$) at week 24.

3.1.6. Effects of GH therapy in patient subgroups

We also investigated the effects of GH on body composition according to gender (male and female) and time of onset of GHD (Table 5). In GH-treated patients, the mean percentage increase in LBM compared with baseline was significant in males (6.4 ± 5.3%) but not females (2.0 ± 3.9%). This difference was also true when the change in LBM with GH treatment was compared with the change in the placebo group. There were no significant differences in changes in serum lipids with gender.

Although GH treatment achieved significant increases in LBM compared with baseline in patients with CO GHD and those with AO GHD, the mean

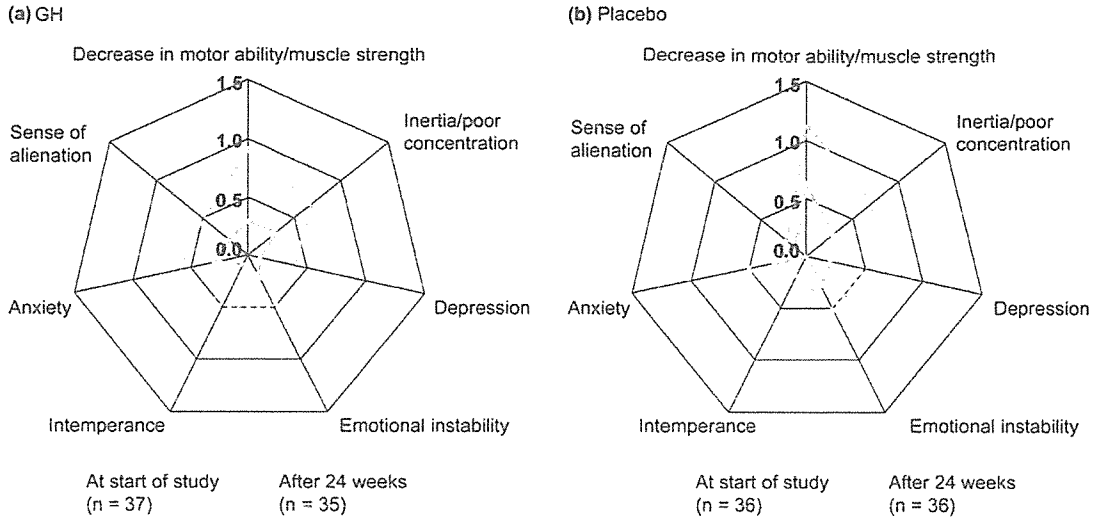

Fig. 2. Subjective and objective symptom scores (mean) at baseline and after 24 weeks in patients who received GH or placebo (ITT population). The severity of subjective and objective symptoms was graded as: 0, absent; 1, mild; 2, moderate; or 3, severe.

Table 5
Mean percent change in lean body mass (LBM) and body fat mass (BFM) and mean change in IGF-I SD score from baseline at week 24 according to gender (male and female) and time of onset of GHD (ITT population)

	GH		Placebo		GH vs placebo[b]
Patient subgroup	*n*	% Change[a]	*n*	% Change[a]	
LBM					
Male	23	6.4 ± 5.3*	23	1.6 ± 4.5*	$p = 0.0006$
Female	14	2.0 ± 3.9	13	0.1 ± 4.1	$p = 0.0893$
Childhood onset	19	4.4 ± 6.3*	19	1.9 ± 4.4	$p = 0.0903$
Adult onset	18	5.1 ± 4.1*	17	0.1 ± 4.3	$p = 0.0008$
BFM					
Male	23	−11.9 ± 13.0*	23	0.6 ± 7.6	$p = 0.0012$
Female	14	−5.1 ± 8.2*	13	−0.4 ± 6.4	$p = 0.1387$
Childhood onset	19	−8.2 ± 12.7*	19	1.3 ± 7.0	$p = 0.0219$
Adult onset	18	−10.4 ± 11.0*	17	−1.0± 7.2	$p = 0.0133$
IGF-I SD score					
Male	23	3.1 ± 1.6*	23	0.0 ± 0.5	$p < 0.0001$
Female	14	2.3 ± 1.7*	13	0.2 ± 0.4	$p = 0.0017$
Childhood onset	19	2.6 ± 1.8*	19	0.0 ± 0.4	$p < 0.0001$
Adult onset	18	2.9 ± 1.4*	17	0.1 ± 0.5	$p < 0.0001$

All values expressed as mean ± SD.
[a] Wilcoxon signed rank test (*$p < 0.05$).
[b] Wilcoxon rank sum test.

absolute and percent changes were greater in those with AO GHD.

GH treatment reduced BFM in all patients compared with baseline, with decreases ranging from 11% to 35%. The mean percent reductions with treatment in female patients were not significantly greater than placebo (Table 5). With GH treatment, mean values of IGF-I SD score increased significantly in all patient subgroups compared with baseline and compared with placebo (Table 5).

3.2. Safety

A total of 265 adverse events were reported in 62 out of 73 patients (84.9%) in the study. GH replacement therapy was well tolerated and the proportion of patients in the active treatment group who experienced adverse events of any cause (86.5%) was very similar to that in the placebo group (83.3%). The most common adverse events were upper respiratory tract infection, muscle weakness and oedema (Table 6). The majority

Table 6
Incidence of most common adverse events during the study period in the ITT population

	GH ($n = 37$), n (%)	Placebo ($n = 36$), n (%)
Upper respiratory tract infection	13 (35.1)	15 (41.7)
Muscle weakness	9 (24.3)	5 (13.9)
Oedema	8 (21.6)	1 (2.8)
Thinking abnormal	7 (18.9)	9 (25.0)
Emotional lability	6 (16.2)	8 (22.2)
Arthralgia	5 (13.5)	2 (5.6)
Anxiety	4 (10.8)	4 (11.1)
Depersonalization	4 (10.8)	5 (13.9)
Depression psychotic	4 (10.8)	6 (16.7)

The table shows the numbers of patients (%) reporting adverse events that occurred in >10% of either patient group.

of adverse events in both treatment groups were of mild or moderate severity.

Adverse events that were considered by the study investigators to be study treatment-related were experienced by 59.5% of patients in the active GH treatment group and 25.0% of those who received placebo. The most common treatment-related events in the GH treatment group were oedema (experienced by 21.6% of patients), arthralgia and muscle weakness (both 10.8%). In the placebo group, the most common treatment-related events were emotional lability (experienced by 8.3% of patients) and hypertonia (5.6%).

During the study period, serious adverse events were reported by 2 patients in the GH treatment group (acute gastroenteritis and sudden deafness in 1 patient and acute gastritis in 1 patient) and by 2 patients in the placebo group (abdominal pain in 1 patient and acute bronchitis in 1 patient). Of these adverse events, deafness in the GH-treated patient and abdominal pain in the placebo-treated patient were considered causally related to the study medication. Two patients – 1 patient with oedema and 1 patient with deafness – in the GH treatment group withdrew from the study due to adverse events. In the patient with deafness, this complaint was still present 3 months after withdrawal from the study.

Analysis of laboratory data showed slight, clinically insignificant changes in alkaline phosphatase, phosphate, thyroid-stimulating hormone, free triiodothyronine, and free thyroxine in some patients. There was no change in patients' mean haemoglobin A_{1c} from baseline to week 24 in either treatment group.

4. Discussion

The syndrome of adult GHD is well characterised in Caucasians [12], and has also been suggested as a similar clinical entity in Japanese patients [6,10]. GHD in adulthood is associated with characteristic pathophysiological changes that include increased BFM, reduced LBM and extra-cellular fluid, and decreased bone density. These changes in body composition, which are accompanied by reductions in muscle strength, energy and in psychological well-being, result in poorer QoL for affected patients. Importantly, adults with GHD are at increased risk of cardiovascular morbidity and mortality. Although Japanese adult patients with GHD are on average less obese than Western populations, the thresholds for obesity-related cardiovascular risk factors, such as hyperglycaemia and dyslipidaemia, are also lower [10].

The beneficial effects of GH replacement therapy, which aims to reverse or normalize the abnormalities associated with GHD, are also well documented in Western populations [13], but again there are few published data on the effects of such treatment in Japanese adults with GHD [10,14]. We conducted this 24-week randomised, controlled clinical trial, in which we compared the effects of recombinant human GH with that of placebo in Japanese adult patients with GHD, to complement the similar study of a different commercially available GH preparation that we recently reported [10].

The results of this study showed that the administration of GH significantly improved body composition, increasing the reduced LBM and reducing the increased BFM. The changes in these outcome measures are of similar magnitude to those we reported previously [10]. GH therapy also significantly reduced total cholesterol and LDL-cholesterol, and significantly increased NEFA. These results are broadly consistent with other national and international study results and demonstrate the beneficial effects of this treatment in reducing the cardiovascular risk factors that are inherent to GHD.

Several retrospective studies have found that increased cardiovascular mortality contributes to the increased overall mortality observed in hypopituitary patients receiving hormone replacement without GH [15–19]. In the general population, central or abdominal obesity, strongly predicts cardiovascular disease, non-insulin dependent diabetes mellitus, stroke and death in men and women [20,21]. The reduction of fat mass in patients with GHD treated with GH is mainly due to a decrease in the visceral fat deposition. Improvements in the serum lipid profile seen in this and other studies result from a GH-induced increase in the expression of hepatic LDL-cholesterol receptors, while the significant increase in NEFA that was observed is due to the action of GH on fatty acid mobilisation [12,22].

In this study, the efficacy of GH therapy is also evident from the significant increases in serum IGF-I levels, but which were virtually unchanged in the placebo group. GH normalized IGF-I levels in many patients whose serum IGF-I values were below the lower limit of age- and sex-matched reference values. The increases in IGF-I were found in all patient subgroups irrespective of gender, age or time of onset of GHD, consistent with our previous reported findings [10].

The causes of GHD in adulthood and the clinical signs of GHD in affected adult patients vary depending on the onset period of the disease [1,23]. Onset of GHD in adulthood is often secondary and of an organic cause, whereas CO GHD is often idiopathic. Patients with CO GHD are characteristically of short stature in childhood and it is not uncommon for some patients to be more than 2 SD below normal at their adult height. In adulthood, patients with CO GHD are typically shorter, smaller and lighter in body weight with less muscle and bone mass, and have a smaller BMI and waist/hip ratio compared with those with AO GHD [1,23]. In this study, we found that administration of GH improved LBM and BFM in patients with both CO and AO GHD, again confirming our previous observations made during a similar study [10]. In this study, as in previous studies that have compared CO GHD and AO GHD [24–26], mean values of IGF-I SD score were below the reference range for age and gender in patients with CO GHD. Despite this, administration of GH significantly increased mean IGF-I concentrations irrespective of time of onset of GHD.

Our results show that body composition improved with GH treatment regardless of age. Other studies have also shown that elderly patients with GHD respond to GH therapy similarly to young and middle-aged patients, with improvements in body composition, bone metabolism and quality of life [27–29]. In terms of gender, however, a significant improvement in body composition was observed in males, with significant improvements in LBM and BFM, but not in females. The reason for this difference is unclear, but it appears that the increase in serum IGF-I during the treatment period, though significant, was less in females than in males. Several studies have shown that the sensitivity of GH-deficient adults to GH is gender dependent [30–32]. This difference is largely due to interactions between sex steroids and the GH/IGF-I axis. Oestrogen in particular attenuates GH action, so that higher doses of GH are needed to reach the same serum IGF-I concentration as men [33]. In this study, GH was administered using a forced incremental method regardless of IGF-I values. If the dose of the agent were adjusted in stages within the standard values based on the IGF-I values of each patient, the gender difference observed in this study is likely to have been reduced.

Mårdh et al. analysed data combined from seven European clinical trials to include 124 patients [34]. All the trials consisted of a 6-month, double-blind, placebo-controlled treatment period followed by open-label treatment for 6 or 12 months. After 6 months, significant improvements were found in patients' energy levels and sleep (assessed by the Nottingham Health Profile) compared with controls, and a relative improvement in well-being, according to total Psychological General Well-Being questionnaire scores. Other studies have shown improvements in Qol-AGDA scores with GH treatment [35–37].

We therefore examined QoL using the Japanese versions of the SF-36 and QoL-AGHDA questionnaires. The Japanese version of SF-36 enquiry form has been confirmed to have high reliability and relevancy through a metrical assessment [38], and has been used in patients with chronic renal failure, chronic hepatitis and inflammatory bowel disease in Japan. The original QoL-AGHDA has also been confirmed to have high reliability and relevancy for adult GHD patients [39]. Administrations of GH and placebo were both associated with significant changes in SF-36 scores of the awareness of general health conditions, vitality and mental health. These changes were not significantly different between GH treatment group and placebo group. The change in mean QoL-AGHDA score was small both in the GH treatment group and in the placebo group and again there were no significant differences between the treatment groups in QoL-AGHDA score at the end of the study. The apparent lack of effect of treatment on QoL may reflect the small numbers of patients for whom QoL measures were evaluated. In addition, the Japanese QoL-AGHDA has a floor effect (i.e. most patients respond to QoL-AGHDA items with the lowest score, which means that it is difficult to detect differences using this questionnaire), and might be more applicable for the measurement of QoL of more severely affected patients [40]. Re-evaluation of this version of the QoL-AGHDA together with adjustment of the items it includes could improve the questionnaire for assessment of patients' needs [40]. It is also recommended that a QoL questionnaire specific for Japanese adults with GHD is created in the near future because translated QoL questionnaires in general may not be sufficiently sensitive to assess QoL accurately in Japanese patients.

The most common adverse events reported in this study, such as oedema and myalgia, are consistent with those reported elsewhere [12,41] and may reflect the antinatriuretic actions of GH. The frequency of these adverse events was not, however, as high as expected. Because such fluid-related effects are more likely to occur during the initial period of GH treatment, consensus treatment guidelines recommend an initial GH dose of 0.15–0.30 mg/day [3]. The initial dose of 0.003 mg/kg/day (0.24 mg/day based on a body weight of 80 kg) used in this trial is well within this guideline range and substantially less than that used in previous randomised controlled trials [12].

With administration of GH in this study following a forced incremental dosing regimen irrespective of serum IGF-I values, IGF-I SD scores were above 2 SD in 14 of 37 patients (37.8%) after 24 weeks of GH treatment. These patients could be considered to have been administered excess GH. However, serum IGF-I levels do not always reflect the GH status of an individual patient

[11], nor do supranormal levels of serum IGF-I identify all patients with clinical evidence of GH excess [42]. In addition, routine monitoring of serum IGF-I in all patients would have compromised blinding of this study. Notably, no adverse event that could pose a particular clinical problem was observed, confirming the excellent safety profile of GH therapy previously reported by clinical studies in Caucasian adult patients. Noting that titration of GH dose is now the preferred approach to GH treatment of adult patients with GHD, we used a GH dose titration regimen to treat the same patients at same clinical centers in a long-term, open-label study that followed this double-blind, placebo-controlled study.

In conclusion, this placebo-controlled double-blind comparative study has demonstrated that GH therapy has significant favourable effects on body composition in adult Japanese patients with GHD compared with the placebo. Treatment with GH results in improvements in LBM, BFM and total cholesterol, with no clinically relevant adverse effects.

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Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH and ACTH in rats

Daisuke Yamamoto [a], Nobuko Ikeshita [a], Rie Daito [a], Elizabeth Henny Herningtyas [b], Keizo Toda [a], Kentaro Takahashi [b], Keiji Iida [b], Yutaka Takahashi [b], Hidesuke Kaji [c], Kazuo Chihara [b], Yasuhiko Okimura [a,*]

[a] *Department of Basic Allied Medicine, Kobe University School of Medicine, Kobe, Japan*
[b] *Division of Endocrinology/Metabolism, Neurology and Hematology/Oncology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan*
[c] *College of Nursing Art and Science, University of Hyogo, Akashi, Japan*

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Abstract

To examine the effect of obestatin, a recently identified peptide derived from preproghrelin, on pituitary hormone secretion, obestatin was administered in anesthetized male rats. Intravenous administration of obestatin did not show any effect on plasma GH, PRL, ACTH and TSH levels. Since obestatin has been reported to have opposite effects of ghrelin in regulating food intake, gastric emptying and intestinal contractility, GH suppressive effect, which is opposite effect of ghrelin, was tested. Intravenous administration of GHRH or GHRP-2, a ghrelin receptor ligand, resulted in a marked plasma GH elevation. However obestatin did not show any effect on GHRH- or GHRP-2-induced GH rise. Furthermore intracerebroventricular administration of obestatin also did not influence plasma GH, PRL, ACTH and TSH levels. These findings suggest that obestatin has no effect on pituitary hormone secretions despite the presence of GPR39, a receptor for obestatin, in the pituitary.

Keywords: Obestatin; GH; PRL; TSH; ACTH; Ghrelin

1. Introduction

Obestatin is a recently found peptide suppressing food intake when administered intraperitoneally or intracerebroventricularly in mice [1]. Moreover, the newly identified peptide decelerates gastric emptying and decreases intestinal contractility [1]. All the actions of obestatin counteract the well-known effects of ghrelin on food intake and gastrointestinal motility. Interestingly obestatin and ghrelin are derived from the same peptide precursor (preproghrelin). Obestatin has been purified from the rat stomach, and has been detected in the circulation, suggesting that this is secreted from the stomach and may have an endocrine function [1].

In addition to the role in food intake [2,3], gastric emptying and intestinal contractility [4], the main role of ghrelin is stimulating pituitary hormones secretion including GH, PRL and ACTH [5–7]. Since the reported actions of obestatin are opposite to those of ghrelin, obestatin might also have a function modulating pituitary hormone secretion. Furthermore, GPR39 cloned as an orphan receptor has been reported to be a obestatin receptor [1] and the receptor is abundant in the pituitary [8]. These findings suggest obestatin may affect pituitary hormone secretion. In this study, we examined the effect of obestatin on pituitary hormone secretion.

2. Materials and methods

2.1. Animals

We used six-week-old male Sprague–Dawley rats weighing 170–190 g and eight-week-old male C57BL6 mice weighing

* Corresponding author. Department of Basic Allied Medicine, Kobe University Graduate School of Medicine, 7-10-2, Tomogaoka, Suma-ku, Kobe 654-0142, Japan. Tel./fax: +81 78 796 4540.
E-mail address: okimuray@kobe-u.ac.jp (Y. Okimura).

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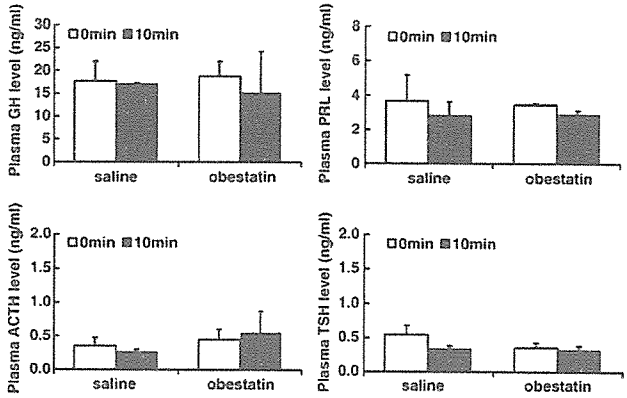

Fig. 1. Effect of intravenous administration of obestatin on pituitary hormone secretion. Plasma GH, PRL, ACTH and TSH levels were not influenced by intravenous administration of obestatin (n=4) as well as saline (n=4) in anesthetized male rats.

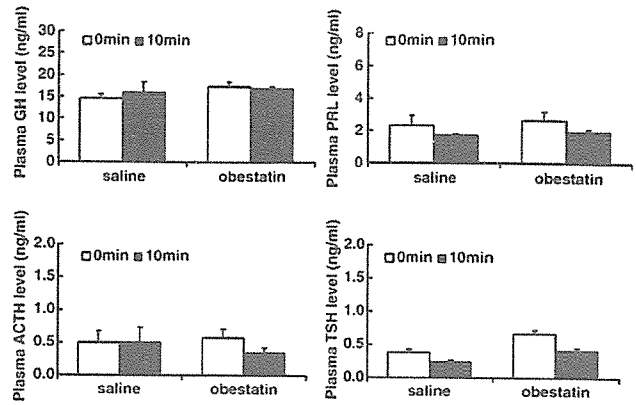

Fig. 3. Effect of intracerebroventricular administration of obestatin on pituitary hormone secretion. Plasma GH, PRL, ACTH, and TSH levels were not influenced by intracerebroventricular administration of obestatin (n=4) as well as saline (n=4) in anesthetized male rats.

21–22 g. Animals were maintained in cages at 22 °C under a 12 h light/12 h dark cycle and were allowed free access to food and water. All the animal protocols were approved by the Committee on Animal Experimentation, Kobe University School of Medicine.

2.2. Obestatin

Obestatin, FNAPFDVGIKLSGAQYQQHGRAL-NH_2 (the active amidated form), was synthesized by Invitrogen. The data sheet shows a single peak in HPLC analysis and >99% purity.

2.3. Effect of intravenous administration of obestatin on pituitary hormone secretion

Eight rats were divided into two groups of four animals each. Under pentobarbital anesthesia (4.0 mg/100 g, i.p.), obestatin (4 nmol/kg) or saline (0.9% NaCl) was administered intravenously. Blood samples were collected from the jugular vein just before and 10 min after administration of obestatin or saline, added EDTA 2Na (1 mg/ml blood) and aprotinin (500 U/ml blood), and centrifuged. The plasma samples were stored at −80 °C until assayed.

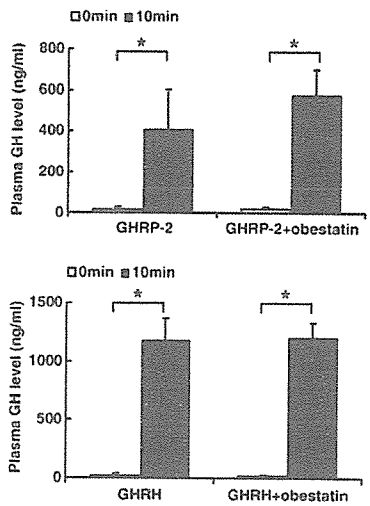

Fig. 2. Effect of intravenous administration of obestatin on GHRP-2- or GHRH-induced GH secretion. Plasma GH levels were markedly increased after the intravenous administration of GHRP-2 or GHRH in anesthetized male rats. However, concomitant administration of obestatin showed no effect on GHRP-2- or GHRH-induced GH secretion. *P<0.05 vs. preinjection group.

2.4. Effect of intravenous administration of obestatin on GHRP-2- or GHRH-induced GH secretion

The inhibitory effect of obestatin on GHRP-2- (a ghrelin receptor ligand) or GHRH-induced GH secretion was examined. Obestatin, GHRP-2 (6 nmol/kg), GHRH (1 nmol/kg) or a combination of these peptides was administered through the jugular vein under pentobarbital anesthesia. Plasma GH was determined before and 10 min after the administrations. Each group contained four rats.

2.5. Effect of intracerebroventricular administration of obestatin on pituitary hormone secretion

Eight rats were divided into two groups of four animals each. Under pentobarbital anesthesia, each rat was immobilized on a stereotactic apparatus. A needle was inserted into a lateral

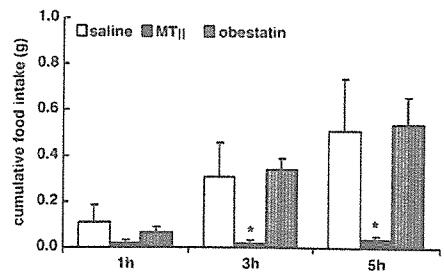

Fig. 4. Effect of intracerebroventricular administration of obestatin on food intake in mice. Intracerebroventricular administration of obestatin did not influence food intake for 5 h while MTII decreased food intake. *P<0.05 vs. saline injection group.

ventricle, and obestatin (4 nmol/kg) or saline was administered through the needle. Blood sampling and storage were the same as described in the above.

2.6. Pituitary hormone assay

Rat GH, PRL, ACTH, TSH, FSH and LH were assayed with rat GH-EIA Kit, rat PRL-EIA Kit (SPI-BIO), rat ACTH EIA Kit (Phoenix Pharmaceuticals), rodent TSH ELISA Test Kit, rodent LH ELISA Test Kit and rodent FSH ELISA Test Kit (Endocrine Technologies), respectively according to the manufacturer's instructions.

2.7. Food intake

Intracerebroventricular guide cannulae were implanted into the lateral cerebral ventricles of mice. Following surgery, mice were housed individually and allowed to recover for 7 days. Mice were acclimated to handling and wire mesh bottom cages. Before intracerebroventricular injection of different peptides, mice were deprived of food for 16 h with free access to water and injections were carried out 2 h after the beginning of the light phase. Obestatin (8 nmol/kg, $n=4$), melanocortin agonists MTII (8 nmol/kg, $n=4$) or saline ($n=4$) was delivered intracerebroventricular. Food intake was measured by placing preweighed pellets in the cage and weighing uneaten pellets at 1, 3, 5 h after intracerebroventricular injection. Proper placement of cannulae was verified at the end of the experiment by dye injection.

2.8. Statistical analysis

Results are expressed as mean±SEM. Differences of hormone level between before and after the treatments were determined by paired T-test. The effect of intracerebroventricular administration of obestatin on food intake was determined by Student's T-test. $P<0.05$ was considered as significant.

3. Results

3.1. Effect of intravenous administration of obestatin on pituitary hormone secretion

Basal GH, PRL, ACTH and TSH levels were 18.8±3.2, 3.5±0.06, 0.45±0.15 and 0.35±0.07 ng/ml, respectively. Plasma GH, PRL, ACTH and TSH levels 10 min after the intravenous administration of obestatin were 15.3±9.0, 2.9±0.3, 0.55±0.32 and 0.32±0.06 ng/ml, respectively. Plasma GH, PRL, ACTH and TSH levels were not influenced by obestatin as well as saline (Fig. 1). FSH and LH levels were below the minimal detection limit in the plasma sample obtained before and after the administration of obestatin.

3.2. Effect of intravenous administration of obestatin on GHRP-2- or GHRH-induced GH secretion

Plasma GH levels were markedly increased after the administration of GHRP-2 or GHRH. The GH levels before and after GHRP-2 administration were 16.8±13.9 and 409±191 ng/ml, respectively. The GH levels before and after concomitant administration of GHRP-2 and obestatin were 19.4±10.2 and 578±123 ng/ml, respectively. The GH levels before and after GHRH administration were 22.5±5.6 and 1180±308 ng/ml, respectively. The GH levels before and after concomitant administration of GHRH and obestatin were 18.9±3.9 and 1206±404 ng/ml, respectively. These results indicated that obestatin had no effect on GHRP-2- or GHRH-induced GH release (Fig. 2).

3.3. Effect of intracerebroventricular administration of obestatin on pituitary hormone secretion

Basal plasma GH, PRL, ACTH and TSH levels were 17.4±1.1, 2.6±0.6, 0.57±0.14 and 0.67±0.05 ng/ml, respectively. Intracerebroventricular administration of obestatin did not influence plasma GH, PRL, ACTH and TSH levels with a mean±SEM levels of 17.2±0.5, 1.9±0.2, 0.35±0.07 and 0.42±0.04 ng/ml, respectively (Fig. 3).

3.4. Food intake

Intracerebroventricular administration of obestatin did not influence food intake for 5 h while MTII decreased food intake (saline, 0.51±0.23; MTII, 0.04±0.02; obestatin, 0.54±0.12 g/5 h; Fig. 4).

4. Discussion

In the present study, obestatin, when administered intravenously or intracerebroventricularly, did not show any influence to basal levels of pituitary hormones. This finding showed that obestatin did not have a stimulating effect of the pituitary hormones. Since obestatin has been reported to be a peptide that has opposite actions to that of ghrelin, we examined its inhibitory action for GH secretion. Obestatin did not show any effect on GH release induced by GHRP-2 or GHRH. The obestatin dose used in these experiments was 4 nmol/kg. Generally hormones show a marked effect in nmol order. For example 3 nmol/kg ghrelin substantially increased GH secretion [9]. Kd of ghrelin receptor for its ligand has been reported to be 1.4–2.6 nM [10]. Since the Kd of obestatin for GPR39 is also 1 nM [1], 4 nmol/kg obestatin seems enough to show effects. Thus it is unlikely that the dose of administered obestatin was too low to influence pituitary hormone secretions.

There are no reports concerning the distribution of GPR39 within the pituitary although the pituitary is abundant in GPR39 mRNA. Also, it is not reported whether the pituitary is rich in GPR39 protein as well as GPR39 mRNA. These might explain the result that obestatin does not give any influence to pituitary hormone secretion despite the presence of GPR39 mRNA in the pituitary.

Intracerebroventricular administration of obestatin did not suppress food intake in mice. We do not know why our results conflicted with previous results [1]. It might be required to reevaluate the inhibitory action of obestatin on feeding.

Acknowledgments

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Growth Hormone-Releasing Hormone (GHRH) Neurons in the Arcuate Nucleus (Arc) of the Hypothalamus Are Decreased in Transgenic Rats Whose Expression of Ghrelin Receptor Is Attenuated: Evidence that Ghrelin Receptor Is Involved in the Up-Regulation of GHRH Expression in the Arc

Asuka Mano-Otagiri,* Takahiro Nemoto,* Azusa Sekino, Naoko Yamauchi, Yujin Shuto, Hitoshi Sugihara, Shinichi Oikawa, and Tamotsu Shibasaki

Departments of Physiology (A.M.-O., T.N., A.S., N.Y., T.S.) and Medicine (Y.S., H.S., S.O.), Nippon Medical School, Bunkyo-ku, Tokyo 113-8602, Japan

GH secretagogue (GHS)/ghrelin stimulates GH secretion by binding mainly to its receptor (GHS-R) on GHRH neurons in the arcuate nucleus (Arc) of the hypothalamus. GHRH, somatostatin, and neuropeptide Y (NPY) in the hypothalamus are involved in the regulatory mechanism of GH secretion. We previously created transgenic (Tg) rats whose GHS-R expression is reduced in the Arc, showing lower body weight and shorter nose-tail length. GH secretion is decreased in female Tg rats. To clarify how GHS-R affects GHRH expression in the Arc, we compared the numbers of GHS-R-positive, GHRH, and NPY neurons between Tg and wild-type rats. Immunohistochemical analysis showed that the numbers of GHS-R-positive neurons, GHRH neurons, and GHS-R-positive GHRH neurons were reduced in Tg rats, whereas the numbers of NPY neurons and GHS-R-positive NPY neurons did not differ between the two groups. The numbers of Fos-positive neurons and Fos-positive GHRH neurons in response to KP-102 were decreased in Tg rats. Competitive RT-PCR analysis of GHRH mRNA expression in the cultured hypothalamic neurons showed that KP-102 increased NPY mRNA expression level and that NPY decreased GHRH mRNA expression level. KP-102 increased GHRH mRNA expression level in the presence of anti-NPY IgG. GH increased somatostatin mRNA expression. Furthermore, GH and somatostatin decreased GHRH mRNA expression, whereas KP-102 showed no significant effect on somatostatin mRNA expression. These results suggest that GHS-R is involved in the up-regulation of GHRH and NPY expression and that NPY, somatostatin, and GH suppress GHRH expression. It is also suggested that the reduction of GHRH neurons of Tg rats is induced by a decrease in GHS-R expression. (*Endocrinology* 147: 4093–4103, 2006)

GH SECRETAGOGUES (GHSs), which were developed from the structure of met-enkephalin (1), strongly stimulate GH secretion by acting on GHRH neurons in the arcuate nucleus (Arc) of the hypothalamus, their main site of action, and on somatotrophs of the pituitary through the GHS receptor (GHS-R), a member of the G protein-coupled receptor superfamily (2). Ghrelin, an endogenous ligand for GHS-R, has been discovered in rat stomach extract (3). GHS-R mRNA is expressed in several hypothalamic nuclei, such as the Arc, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, supraoptic nucleus, suprachiasmatic nucleus, lateroanterior hypothalamic nucleus, and tuberomammillary nucleus, and other brain areas such as the hippocampus, substantia nigra, ventral tegmental area, and dorsal and median raphe nuclei (4). The systemic administration of GHSs induces c-*fos* mRNA expression in the neurons of Arc. Overall, of these c-*fos* mRNA-expressing neurons, 51% are neuropeptide Y (NPY) neurons, 23% are GHRH neurons, 11% are tyrosine hydroxylase (TH) neurons, 11% are proopiomelanocortin neurons, and 4% are somatostatin neurons (5, 6).

To clarify the physiological significance of GHS-R, we have created transgenic (Tg) rats expressing an antisense GHS-R mRNA that is designed to be specific for the region around the initiation codon of GHS-R, under the control of the promoter for TH (7). TH is a rate-limiting enzyme in catecholamine biosynthesis and is a marker for the dopaminergic neurons. TH is present in most neurons in the ventral portion of the Arc where GHRH neurons exist (8). TH mRNA-expressing neurons in the Arc of Tg rats have been shown to express antisense GHS-R mRNA (7). GHS-R mRNA is detected in NPY and GHRH neurons in the Arc (9–11). We have found that GHS-R is expressed in both GHRH and NPY neurons in the Arc of Tg rats and slc:SD wild-type (WT) rats and that GHS-R, as determined by Western blot analysis, in the Arc is reduced in Tg rats compared with WT rats (7). These Tg rats have lower body weight and

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* A.M.-O. and T.N. contributed equally to this work.

Abbreviations: Arc, Arcuate nucleus; DNase, deoxyribonuclease; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; ICV, intracerebroventricular; NPY, neuropeptide Y; PeV, periventricular nucleus; Tg, transgenic; TH, tyrosine hydroxylase; WT, wild type.

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less adipose tissue, suggesting that GHS-R plays a role in the regulation of adiposity (7). They also have lower GH responses to iv administered KP-102, one of the GHSs, than WT rats (7). Furthermore, GH secretion and plasma insulin-like growth factor I levels are significantly reduced in female Tg rats, suggesting that GHS-R is involved in the regulation of GH secretion and that GHS-R plays a more important role in the regulatory mechanism of GH secretion in female than in male rats (7). However, the details of GHRH, GHS-R, and NPY expression in the Arc of Tg rats have not yet been examined. The relation of the GHS-R expression level with the expression of GHRH and NPY in the Arc has also not yet been studied. Recently, it has been reported that Tg mice overexpressing GHS-R1A in the GHRH neurons show an increase in hypothalamic GHRH expression (12), suggesting that GHS/GHS-R may regulate GHRH expression.

Therefore, in this study, we first compared the numbers of GHRH neurons, NPY neurons, GHS-R-positive neurons, GHS-R-positive GHRH neurons, and GHS-R-positive NPY neurons located in the Arc of Tg and WT rats to clarify whether the GHS-R expression levels affect GHRH and NPY expression. We then examined Fos expression of the neurons located in the Arc of Tg and WT rats in response to intracerebroventricular (ICV) injection of KP-102 to confirm that GHS-R is reduced in the Arc of Tg rats. We finally examined the effects of KP-102, NPY, somatostatin, and GH on the GHRH mRNA, NPY mRNA, or somatostatin mRNA expression level in primary cultured hypothalamic neurons of normal rats to clarify whether and how ghrelin/GHS-R affects the expression level of GHRH mRNA.

Materials and Methods

Animals

Twelve-week-old random-cycling female Tg rats (7) and WT rats were used in this study. The WT rats used in the present study were not littermates of Tg rats, although we used WT rats, which were littermates of Tg rats, in our previous studies (7). WT rats, which we used for these 5 yr, always showed similar results. The Tg rats, which are homozygote for transgene, also showed unchanged phenotype. There were significant differences in body weights [Tg rats (n = 14), 199.6 ± 2.6 g *vs.* WT rats (n = 14), 215.6 ± 2.3 g, $P < 0.001$] and in nose-tail length [Tg rats (n = 14), 36.9 ± 0.2 cm *vs.* WT rats (n = 14), 37.5 ± 0.2 cm, $P < 0.05$]. All animals were housed under controlled temperature and illumination (0800–2000 h) and allowed *ad libitum* access to food and water. To administer samples ICV, a polyethylene cannula was implanted into the right lateral ventricle under sodium pentobarbital anesthesia (50 mg/kg body weight ip injection), as described previously, 5 d before the experiment was to be done (13). All experimental procedures were conducted in accordance with the guidelines on the use and care of laboratory animals approved by the Local Ethics Committee of Nippon Medical School, Japan.

Effect of ICV injection of KP-102 on Fos expression

On the day of the experiment, rats with *ad libitum* access to food and water were given vehicle (2 μl saline) or KP-102 (100 pmol/2 μl) ICV. Ninety minutes after the injection, the rat brain was fixed for immunohistochemistry.

Immunohistochemistry of GHS-R, GHRH, and NPY

To compare the numbers of GHRH neurons, NPY neurons, and GHS-R-positive neurons of Tg and WT rats, the rats were injected ICV with colchicine (100 μg/5 μl saline) through the cannula. Tg and WT rats were anesthetized with pentobarbital (50 mg/kg body weight ip injection) and perfused via an intracardiac cannula with PBS followed by 4% paraformaldehyde 48 h after the injection of colchicine. The brain was removed, left overnight in 4% paraformaldehyde and then transferred to 20% sucrose/PBS. Coronal sections (20 μm) were cut with a cryostat and mounted onto gelatinized slides. Successive sections were used for immunohistochemistry of GHS-R, GHRH, and NPY.

Immunohistochemistry was performed with the avidin-biotin-peroxidase method using specific antiserum against rat GHRH (14), mouse GHS-R (15), and rat/human NPY (16). Briefly, sections were incubated with specific polyclonal antiserum against GHRH (1:1000), GHS-R (1:500) (7), or NPY (1:1000) overnight at 4 C. The tissues were then rinsed in PBS and incubated in biotinylated goat antirabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. This was followed by another 1-h incubation in avidin-biotin complex solution (Vectorstain ABC Elite kit, Vector Laboratories) at room temperature. The reaction product was visualized using a nickel-intensified diaminobenzidine reaction that gives a dark-brown precipitate. Briefly, sections were washed in 0.1 M sodium acetate buffer (pH 6.0) and incubated in the same buffer containing 2.5% nickel sulfate, 0.2% glucose, 0.04% ammonium chloride, 0.025% diaminobenzidine, and 30 U/ml glucose oxidase (Sigma-Aldrich, St. Louis, MO). The reaction was stopped by washing in 0.1 M acetate buffer under microscopic observation (17). The tissue was dehydrated through graded alcohols, cleared in xylenes, and then coverslipped with Vector Mount (Vector Laboratories). Preincubation of anti-GHRH serum with 1.0 μg GHRH, anti-GHS-R serum with 1.0 μg GHS-R, and anti-NPY serum with 1.0 μg NPY completely abolished the staining of GHRH, GHS-R, and NPY, respectively. Quantita-

TABLE 1. Primer sequences of the studied genes

Genes	Primers (5′–3′)	Length (bp)	GenBank accession no.
GHRH			
Sense	ATGCCACTCTGGGTGTTCTTTG	315	NM_031577
Antisense	TCAAGCCTCCGCTGAAAGCTTC		
Competitor sense	ATGCCACTCTGGGTGTTCTTTGGCCATGCAGACGCCATCTTCAC	211	
Competitor antisense	TCAAGCCTCCGCTGAAAGCTTCTCTGCTTGTCCTCTGCCCAC		
NPY			
Sense	CCGCTGCGCAGAGACCACAGCC	405	NP_036746
Antisense	TCAGACTGGTTTCACAGGATG		
Competitor sense	CCGCTGCGCAGAGACCACAGCCGCTCGTGTGTTTGGGCATTC	254	
Competitor antisense	TCAAGCCTCCGCTGAAAGCTTCTCTGCTTGTCCTCTGCCCAC		
Somatostatin			
Sense	ATGCTGTCCTGCCGTCTCCAGTG	351	NM_012659
Antisense	CTAACAGGATGTGAATGTCTTCCAG		
Competitor sense	ATGCTGTCCTGCCGTCTCCAGTGTCTGCATCGTCCTGGCTTTG	212	
Competitor antisense	CTAACAGGATGTGAATGTCTTCCAGGGCATCGTTCTCTGTCTGGTTG		
β-Actin			
Sense	TCATGAAGTGTGACGTTGACATCCGT	285	BC063166.1
Antisense	CCTAGAAGCATTTGCGGTGCACCGATG		

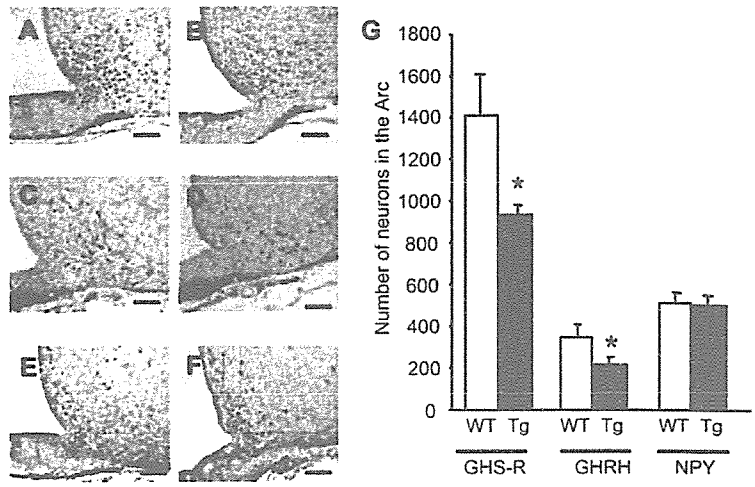

FIG. 1. Localization GHS-R-positive neurons, GHRH neurons, and NPY neurons in the Arc of female WT and Tg rats. GHS-R-positive neurons of female WT and Tg rats are shown in A and B, respectively, GHRH neurons of female WT and Tg rats in C and D, respectively, and NPY neurons of female WT and Tg rats in E and F, respectively. *Scale bars*, 100 μm. The numbers of GHS-R-positive neurons, GHRH neurons, and NPY neurons in the Arc are shown in G. *, $P < 0.05$ *vs.* WT rats. The number of rats of each group was five.

tive analysis of the number of GHS-R, GHRH, and NPY neurons was performed using an image analysis system (MCID Amersham Biosciences, Tokyo, Japan). Under light microscopy, at 200× magnification, the total number of positive neurons was counted in the Arc. Every three sections of 20 μm were used for the counting of GHS-R, GHRH, or NPY (eight sections for GHRH, GHS-R, and NPY, respectively, per rat).

Double-labeled immunohistochemistry for GHS-R with GHRH or NPY

Double-labeled immunofluorescence for GHS-R and GHRH or NPY coupled with confocal microscopic analysis was done using hypothalami of colchicine-treated rats. Coronal sections (10 μm) were mounted onto gelatinized slides. Sections were incubated in antiserum against GHS-R (1:500) overnight at 4 C. The tissues were then rinsed in PBS and incubated in fluorescein-conjugated goat antirabbit IgG (1:200, Vector Laboratories) for 3 h at room temperature. The sections were washed in PBS and subsequently incubated overnight at 4 C with the second antibody, GHRH or NPY. After washing in PBS, the tissues were incubated in Texas red-conjugated goat antirabbit IgG (1:200; Vector Laboratories) for 3 h at room temperature. The slides were coverslipped with VECTASHIELD Hard Set mounting medium (Vector Laboratories). Sections were examined using a Zeiss LSM 510 confocal microscope (Carl Zeiss Co. Ltd., Thornwood, NY). Immunofluorescence in tissue sections was visualized by using a Zeiss Axioplan photomicroscope with a multiband filter set for independent or simultaneous visualization of fluorescein (excitation range, 447–501 nm; emission range, 510–540 nm) and Texas red (excitation range, 560–596 nm; emission range, 610–655 nm) fluorophores. Double-labeled neurons for GHS-R and GHRH or NPY in the Arc were counted in five randomly selected sections under light microscopy at ×400 magnification. All images were processed by using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Immunohistochemistry for Fos

Twenty serial coronal sections (40 μm) were cut with a cryostat through the Arc. Immunohistochemistry was done using the free-floating method with the avidin-biotin-peroxidase method using Fos antibody (1:10000; rabbit polyclonal, Ab5; Oncogene, San Diego, CA). The antibody-peroxidase complex was visualized using diaminobenzidine (Vector DAB kit, Vector Laboratories).

Double-labeled immunohistochemistry for Fos and GHRH

Forty-micrometer sections were processed for immunohistochemical detection of Fos using the Vector DAB kit, and they were then incubated with GHRH antiserum (1:2000) overnight at room temperature. Immunostaining was visualized using the Vector SG substrate kit (Vector Laboratories), which gives a blue-gray precipitate. Quantitative analysis of the number of Fos-positive neurons was done using MCID image analysis system (MCID Amersham Biosciences). Under light microscopy at ×200 magnification, the total number of Fos-positive neurons was counted in the Arc. In total, nine sections per Arc were used for counting. Double-labeled neurons for Fos and GHRH in the Arc were counted in five randomly selected sections under light microscopy at ×400 magnification. Sections were viewed and photographed with an Olympus AX 80 microscope. All images were processed by using Adobe Photoshop software (Adobe Systems).

Primary culture of rat hypothalamic neurons

Newborn Wistar rats were killed by decapitation, and their hypothalami were removed under sterile conditions. Hypothalami were minced in a 1:1 mixture of DMEM and Ham's nutrient mix F-12 containing 10% fetal calf serum, penicillin, and streptomycin (DMEM/F12) (Sigma-Aldrich) for suspension. The minced tissues were then washed twice in PBS. The mixture was incubated in PBS containing 0.047 g/liter $MgCl_2$, 0.1 g/liter $CaCl_2$, and 0.01% Dispase (Godoshusei, Tokyo, Japan) with constant stirring for 30 min at room temperature. After being washed three times with PBS, 1-ml aliquots of cell suspension containing 5.0×10^4 cells in a DMEM/F12 were placed in the wells of 24-well plates that were coated with poly-D-lysine (Sigma-Aldrich). The cells were subsequently allowed to attach to the bottom surface in a humidified 95% air-5% CO_2 incubator for 6 d.

To test the effect of KP-102 on GHRH mRNA expression, cells were treated with KP-102 at concentrations ranging from 2–200 nM for 2 h, and cells were also treated with 20 nM KP-102 for 1–24 h to test the time course of the effect. To test the effect of KP-102 on NPY or somatostatin mRNA expression, cells were treated with 0.2, 2.0, or 20 nM KP-102 for 2 h or 20 nM KP-102 for 24 h.

To test the effect of NPY on GHRH mRNA expression, cells were treated with NPY at a concentration of 0.1 or 1.0 nM for 2 h. To delete the influence of NPY on KP-102-induced changes in GHRH mRNA expression in the culture system, anti-NPY IgG was used. To examine the binding capacity of anti-NPY IgG to NPY in the culture system, cells were treated for 2 h with 1 nM NPY plus anti-NPY IgG (3.6 μg/ml) or normal rabbit serum IgG (3.6 μg/ml) prepared from rabbit anti-NPY serum (16) or normal rabbit serum, respectively, using Protein A Sepharose 4FF (Pharmacia Biotech, Tokyo, Japan). Subsequently, the expression of GHRH mRNA was determined. To examine the effect of anti-NPY IgG on KP-102-induced changes in GHRH mRNA expression, cells were incubated with KP-102 plus anti-NPY IgG or normal rabbit serum IgG (3.6 μg/ml) for 2 h. To examine the effect of anti-NPY IgG on somatostatin-induced changes in GHRH mRNA expression, cells were incubated with 10 nM somatostatin plus anti-NPY IgG or normal rabbit serum IgG for 2 h.

To test the effect of somatostatin on GHRH mRNA expression, cells were treated with somatostatin at a concentration of 1, 10, or 100 nM for 2 h. To delete the influence of somatostatin on the KP-102-induced change in the GHRH mRNA expression level in the culture system, antisomatostatin IgG was used. Antisomatostatin serum was obtained from female New Zealand white rabbits by immunizing them with

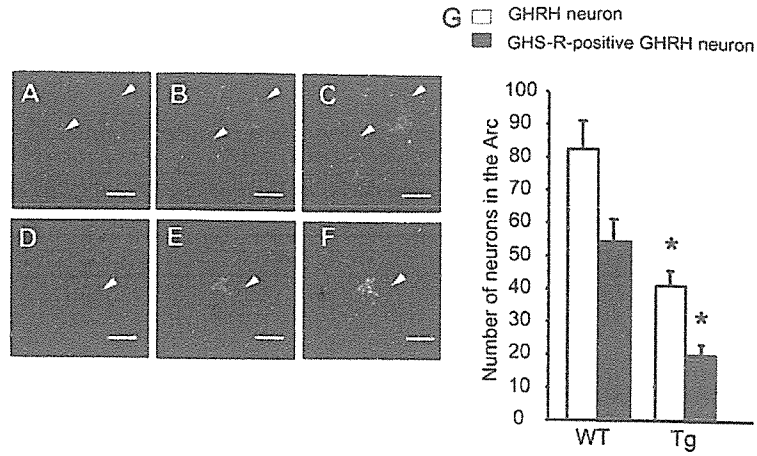

FIG. 2. Double-labeled immunohistochemistry of GHS-R and GHRH in the Arc of female WT and Tg rats. Confocal images of GHS-R (A), GHRH (B), and both (C) of WT rats and those of GHS-R (D), GHRH (E), and both (F) of Tg rats. *Arrows*, Positive neurons. G, Numbers of GHRH and GHS-R-positive GHRH neurons in the Arc. *Scale bars*, 10 μm. *, $P < 0.05$ *vs.* WT rats. The number of rats of each group was seven.

synthetic rat somatostatin-14 coupled with porcine thyroglobulin through water-soluble carbodiimide hydrochloride. The antiserum was used in the RIA at a final concentration of 1/380,000 and showed no cross-reactivity with GHRH, NPY, or corticotropin-releasing factor. The antisomatostatin IgG fraction was prepared using Protein A Sepharose 4FF (Pharmacia Biotech). To test the binding capacity of antisomatostatin IgG to somatostatin in the culture system, cells were treated with somatostatin (10 nM) plus antisomatostatin IgG (3.6 μg/ml) or normal rabbit serum IgG (3.6 μg/ml) for 2 h, and then the level of GHRH mRNA expression was determined. To examine the effect of antisomatostatin IgG on KP-102-induced changes in GHRH mRNA expression, cells were incubated with KP-102 plus antisomatostatin IgG or normal rabbit serum IgG (3.6 μg/ml) for 2 h. To examine the effect of somatostatin on NPY mRNA expression, cells were treated with somatostatin at a concentration of 10 nM for 2 h.

To test the effect of GH on GHRH, NPY, or somatostatin mRNA expression, cells were treated with human recombinant GH (ProSpec-Tany TechnoGene LTD, Rehovot, Israel) at concentrations ranging from 1–500 ng/ml for 2 h.

To test the effect of KP-102, NPY, or somatostatin on GHRH synthesis and release, cells were treated with KP-102 at concentrations ranging from 2–200 nM, NPY, or somatostatin at concentrations of 1 and 10 nM for 4–24 h.

RT-PCR

Total RNA was extracted from cells using Isogen according to the manufacturer's instructions (Takara, Shiga, Japan). To avoid false-positive results caused by DNA contamination, a deoxyribonuclease (DNAse) treatment for 60 min at 37 C using ribonuclease-free DNase (Takara) was done. First strand cDNA was synthesized using 1 μg denatured total RNA under conditions of 42 C for 30 min, 99 C for 5 min, and 5 C for 5 min using RT-PCR kit (Takara). PCR was carried out under conditions of denaturation at 94 C for 10 sec, annealing at 50 C for 5 sec, and extension at 72 C for 60 sec for 30 cycles, using specific primers for GHRH, NPY, and somatostatin (Table 1). After amplification, the PCR products were subjected to 2% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, and were then visualized under UV illumination. All PCR-amplified DNAs were sequenced for purposes of confirmation.

Competitor construction

Homologous competitive internal standards that shared the same primer binding sites but contained a shortened internal sequence with respect to the endogenous target RNA for GHRH, NPY, or somatostatin were prepared as follows. The products resulting from PCR were subcloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The cloned pGEM-T vector was linearized by *Nco*I restriction digestion and transcribed into the cRNA template by SP6 RNA Polymerase (Promega). The DNA template was removed after transcription, and the cRNA product was quantified and used as an internal standard in RT-PCR for GHRH, NPY, and somatostatin gene expression.

Competitive RT-PCR

After DNase treatment, the amount of mRNA present in the samples was normalized using β-actin primers as an internal reference standard (Table 1). To test for possible pseudogene or genomic DNA contamination, either the RT enzyme or RNA was omitted from the reaction tube. To confirm that RNA competitor is not contaminated with DNA, we performed RT-PCR using only RNA competitor (10^9 copies) at the maximum amount. The reaction mixture and RNA competitor were added to each tube. RT reaction was carried out under conditions of 42

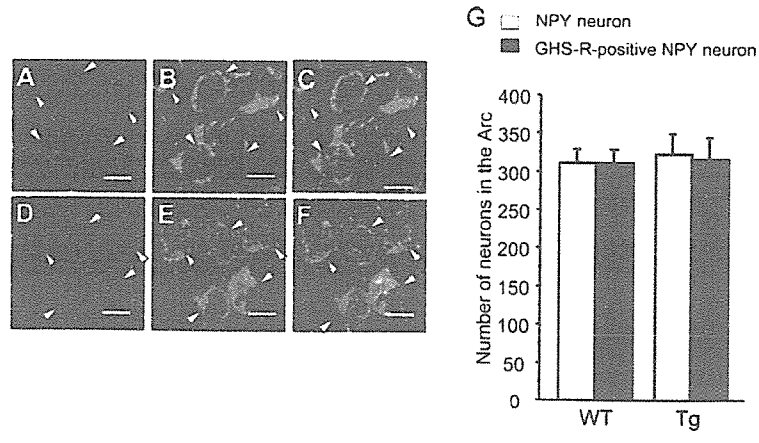

FIG. 3. Double-labeled immunohistochemistry of GHS-R and NPY in the Arc of female WT and Tg rats. Confocal images of GHS-R (A), NPY (B), and GHS-R-positive NPY neurons (C) of WT rats and GHS-R (D), NPY (E), and GHS-R-positive NPY neurons (F) of Tg rats. *Arrows*, Positive neurons. The numbers of NPY and GHS-R-positive NPY neurons in the Arc are shown in G. *Scale bars*, 10 μm. The number of rats of each group was seven.

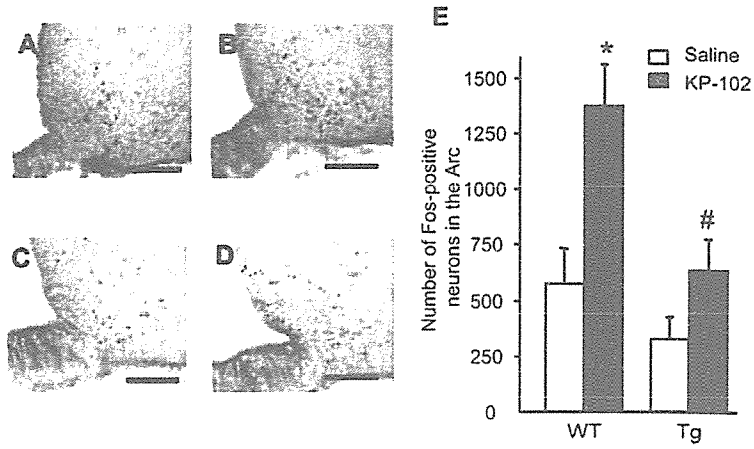

FIG. 4. Changes of Fos-expressing neurons in the Arc of female WT and Tg rats after KP-102 administration. Fos-positive neurons in the Arc 90 min after ICV administration of 2 μl saline (A and C) and 100 pmol KP-102 (B and D) were immunohistochemically determined in WT (A and B) and Tg (C and D) rats. *Scale bars*, 200 μm. E, Statistical analysis of the Fos-positive neurons in the Arc of WT and Tg rats. *, $P < 0.01$ *vs.* WT rats treated with saline; #, $P < 0.01$ *vs.* WT rats treated with KP-102. The number of rats of each group was seven.

C for 30 min, 99 C for 5 min, and 5 C for 5 min. Then, PCR mixture was dispensed into each tube, which contained RT reactant. PCR was carried out under the following conditions: denaturation at 94 C for 10 sec, annealing at 60 C for 5 sec, and primer extension at 72 C for 60 sec for 30 cycles. PCR products were separated on 2% agarose gel and visualized with ethidium bromide. The intensities of the bands of the PCR products of GHRH, NPY, and somatostatin were quantified using National Institutes of Health image software. The ratio of internal standard to endogenous area was plotted as a function of the competitor concentration added to each PCR. The concentrations of GHRH, NPY, and somatostatin mRNA were determined at the point where the ratio of the internal standard and the endogenous area of each gene were equal to 1 (the equivalence point). Experiments of same protocol were repeated twice or three times, and the results were combined for statistical analysis.

RIA for GHRH

RIA for GHRH was performed as described previously (14). In short, synthetic rat GHRH was iodinated using the chloramine-T method and purified on a column of Sephadex G-50. PBS [0.1 M (pH 7.5)] containing 0.01% Nonidet P-40 (Nacalai Inc., Kyoto, Japan), 5 mM EDTA-Na, and 0.02% NaN_3 was used for RIA. Standard synthetic rat GHRH or sample was incubated with antirat GHRH antiserum in 3-ml plastic tubes for 24 h at 4 C. ^{125}I-labeled GHRH was then added to each tube and incubated for another 24 h. Goat antirabbit IgG was used to separate tracer bound to antiserum from free tracer. The anti-GHRH antiserum was used for RIA at a final concentration of 1:400,000 to yield a maximum binding of approximately 30%.

Image analysis

Image analysis was performed with an Olympus AX-80 microscope and a digital camera (DP50, Olympus, Tokyo, Japan). Images were assembled using Lumina Vision (Mitani Corp., Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SEM. The statistical analysis was completed using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA); for *in vivo* study, one-way ANOVA followed by a *post hoc* Fisher's test was performed, whereas for the *in vitro* study, ANOVA followed by Fisher's PLSD was done. $P < 0.05$ considered statistically significant.

Results

Distribution and number of GHS-R-positive, GHRH, and NPY neurons in the Arc

GHS-R-positive neurons were widely distributed in the Arc of the WT and Tg rats (Fig. 1, A and B). The mean number of GHS-R-positive neurons was significantly less in Tg rats than in WT rats (Tg rats, 927 ± 57 *vs.* WT rats, 1409 ± 199, $P < 0.05$) (Fig. 1G). GHRH neurons were distributed in the ventral and lateral part of the Arc of the WT and Tg rats (Fig. 1, C and D). The mean number of GHRH neurons was significantly less in Tg rats than that in WT rats (Tg rats, 206 ± 33 *vs.* WT rats, 326 ± 59, $P < 0.05$) (Fig. 1G). NPY neurons

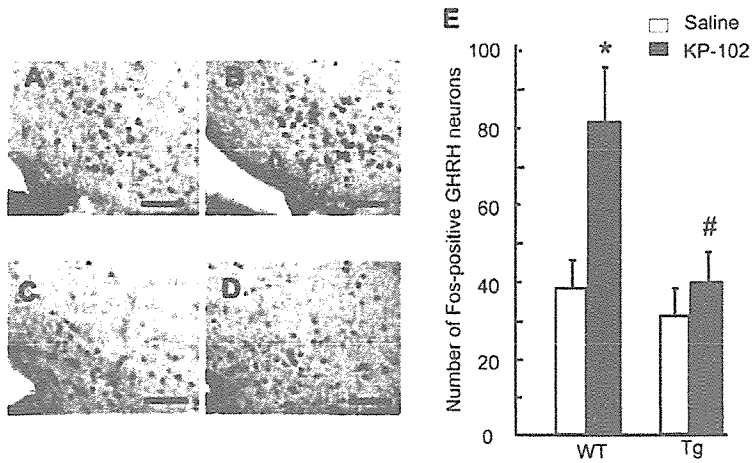

FIG. 5. Distribution and number of Fos-positive GHRH neurons in the Arc in response to saline or KP-102 in female WT and Tg rats. A, Fos-positive GHRH neurons in the Arc 90 min after ICV administration of 2 μl saline (A and C) and 100 pmol KP-102 (B and D) were immunohistochemically determined in WT (A and B) and Tg (C and D) rats. *Scale bars*, 100 μm. E, Statistical analysis of the Fos-positive GHRH neurons in the Arc of WT and Tg rats. *, $P < 0.05$ *vs.* WT rats treated with saline. #, $P < 0.05$ *vs.* WT rats treated with KP-102. The number of rats of each group was eight.