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Ghrelin concentrations and cardiac vagal tone are decreased after pharmacologic and cognitive–behavioral treatment in patients with bulimia nervosa

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Abstract

Patients with bulimia nervosa (BN) have bulimic and depressive symptoms, which have been associated with abnormalities in the neuroendocrine and vagal systems. Subjects included twenty-four female drug-free outpatients with BN that were selected from patients seeking treatment for eating behavior in our hospital along with twenty-five age-matched healthy females who served as controls. We investigated ghrelin and leptin levels, cardiac vagal tone and sympathovagal balance, frequency of sets of binge-eating and vomiting episodes per week and the Profile of Mood States (POMS) depression scale in BN before and after a 16-week administration of the serotonin selective reuptake inhibitor (SSRI) paroxetine combined with cognitive–behavioral therapy. Compared to controls, the BN group had higher ghrelin levels and resting cardiac vagal tone, and lower leptin levels and resting cardiac sympathovagal balance before treatment, although there was a significant difference between the two groups for the body mass index (BMI). The elevated ghrelin levels (301.7 ± 18.9 pmol/l, mean ± SEM vs. 202.8 ± 15.6 pmol/l, $P < 0.01$), cardiac vagal tone (2246.4 ± 335.5 ms^2 vs. 1128.5 ± 193.3 ms^2, $P < 0.01$), frequency of sets of binge-eating and purging episodes and T scores for the POMS depression scale were all significantly decreased after treatment despite similar BMI, percent body fat and leptin levels. In close association with cardiac vagal function and ghrelin recoveries, abnormal eating behavior and depressive symptoms improved, indicating the usefulness of these indexes in the assessment of clinical condition and therapeutic efficacy in BN.
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Keywords: Ghrelin; Leptin; Cardiac vagal tone; Bulimia nervosa

Introduction

Ghrelin was originally discovered in the rat and human stomach and stimulates growth hormone secretion (Kojima et al., 1999; Takaya et al., 2000), increases food intake (Tschöp et al., 2000; Asakawa et al., 2001) and enhances appetite (Wren et al., 2001). This is an orexigenic and gastrointestinal peptide that antagonizes leptin action (Inui, 2001) and which has a role in the regulation of eating behavior and energy metabolism in the central nervous system (Shintani et al., 2001; Nakazato et al., 2001) via the vagal system (Masuda et al., 2000; Asakawa et al., 2001; Date et al., 2002). Recent studies have found that fasting ghrelin levels in patients with bulimia nervosa (BN) are increased (Tanaka et al., 2002; Kojima et al., 2005; Fassino et al., 2005). In addition, we have shown that increased ghrelin concentrations may be associated with binge-eating and vomiting behavior (Tanaka et al., 2003a, 2004). A few studies have shown that BN patients have decreased fasting leptin concentrations (Jimerson et al., 2000; Brewerton et al., 2000) and elevated cardiac vagal tone (Kennedy and Heslegrave, 1989; Rissanen et al., 1998), which suggest that BN patients may have neuroendocrine and vagal system abnormalities. In addition, Rissanen et al. (1998) have shown that the use of the serotonin selective reuptake inhibitor (SSRI) fluoxetine combined with self-monitoring that is based on cognitive–behavioral treatment

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normalizes the elevated cardiac vagal tone in BN patients. Furthermore, BN patients have been reported to respond to treatment with the SSRI fluoxetine (Fluoxetine Bulimia Nervosa Collaborative Study Group, 1992; Goldbloom and Olmsted, 1993; Goldstein et al., 1995). The aim of this study was to examine ghrelin and leptin levels and cardiac vagal tone in subjects before and after a pharmacologic treatment program, which administered SSRI paroxetine in combination with cognitive–behavioral therapy.

Methods

Subjects

Twenty-four female BN patients between the ages of 19 and 32 years old (23.6 ± 0.8 years, mean ± SEM) and twenty-five age-matched (23.1 ± 0.6 years) apparently healthy female volunteers (controls) were enrolled as subjects in this study. BN patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), and all subjects were outpatients examined at Kagoshima University Hospital between April 2002 and August 2003. Patients were excluded if they had a history of alcohol and substance abuse or past anorexia nervosa. Two patients were removed from the study due to non-compliance associated with side effects. Previous treatment histories, including medications, were not used as exclusionary criterion. At the time of initial assessment, all patients had been medication-free for at least 3 months and, once enrolled, did not take any prescription medications until commencement of the drug therapy. Bulimia diagnosis was confirmed by a structured clinical interview (First et al., 1995), which was administered at entry. Information about medical history, age at onset (20.5 ± 0.8 years), duration of illness (3.1 ± 0.4 years) and eating behavior was collected. Patients had habitual sets of binge-eating and vomiting at least twice a week over the preceding 3 months and were excluded if only purging behavior without vomiting was present. Control subjects were recruited from the local community and were interviewed about medical history and food intake over the week prior to initiation of the study. All controls had no history of eating and mental disorders and ate normal diets. In all subjects, body weight, height, percent body fat (Bolanowski and Nilsson, 2001), heart rate variability, plasma ghrelin and glucose, serum leptin, GH, amylase and potassium measurements were performed. BMI was calculated as weight (kg)/height squared (m^2). The Institutional Committee of Kagoshima University approved the study, and all subjects provided written informed consent prior to initiation of the study.

For 16 weeks, patients took 20 mg of paroxetine daily prior to going to sleep. Patients were monitored for side effects that occurred over a 2-week period, including nausea, sleepiness, dry mouth, constipation, blood pressure, tachycardia and sweating. In addition to the medical treatment, patients had individual 50 min cognitive–behavioral therapy sessions (Fairburn et al., 1993). Serum total amylase (Metzger et al., 1999) and potassium (Wolfe et al., 2001) concentrations were measured to determine the severity of binge-eating and purging. Both before and after 16 weeks of treatment, Profile of Mood States (POMS) was used to evaluate associated depressive symptoms. After 16 weeks of treatment, BMI, percent body fat, frequency of sets of binge-eating and purging, heart rate variability, plasma ghrelin and glucose, serum leptin, GH, amylase and potassium were measured and used to determine treatment efficacy.

Sampling and analysis

Blood samples from all subjects were collected between 8:00 and 10:00 AM after an overnight fast. Serum leptin concentrations were measured using an enzyme immunoassay (EIA) kit (LINCO Research, Inc., St. Louis, MO, USA). Serum GH concentrations were measured using a radioimmunoassay (RIA), with reagents provided by Daiichi Radioisotope Laboratories, Ltd. (Tokyo, Japan). Plasma glucose was measured by using a glucose autoanalyzer (Hitachi 7170 Autoanalyzer; Hitachi Ltd., Tokyo, Japan). Serum total amylase was measured using an EIA kit by AZ well corporation (Osaka, Japan). Serum potassium was measured using an ion-selective electrode method. Other blood samples were drawn into chilled tubes containing EDTA·2Na (1 mg/ml) and aprotinin (500 U/ml). The C-terminal region of plasma ghrelin was measured using RIA, as described elsewhere (Shiiya et al., 2002).

Before and after completing the 16-week treatment, subjects were placed in a supine position in a quiet room, and after a 10-min rest period, the surface electrocardiogram was continuously monitored for 10 min. Heart rate variability was assessed by the magnitude of individual components of the heart rate power spectral analysis (Akselrod et al., 1981; Hayano et al., 1990). The power spectral density includes a high-frequency (HF) component at the respiratory frequency and a low-frequency (LF) component at 0.03 to 0.15 Hz (Akselrod et al., 1981; Pagani et al., 1986). We calculated the mean amplitudes for the HF (0.20 to 0.30 Hz) and for the LF (0.03–0.15 Hz). The magnitudes of these components respectively provide indexes of cardiac vagal tone (HF) and cardiac sympathovagal balance (LH/HF) (Pagani et al., 1986; Bigger et al., 1995) with vagal modulation.

Statistical analysis

The subject groups (mean ± SEM) were compared using ANOVA and a post hoc Fisher's test because distributions of the data on BMI and percent body fat were examined for normality. Since the rest of the data distributions were skewed, a Kruskal–Wallis one-way ANOVA with a chi-square statistic was used to test for group differences for the other variables.

Table 1
Comparison of physiological data across study groups (mean ± SEM)

	Bulimia nervosa patients		Age-matched control group	Kruskal–Wallis test	*P* value
	Before treatment	After treatment			
Body mass index (kg/m^2)	18.5 ± 0.4*	19.0 ± 0.4*	21.4 ± 0.3	–	–
Percent body fat (%)	20.8 ± 0.7*	21.7 ± 0.8*	25.9 ± 0.7	–	–
Cardiac vagal tone (ms^2)	2246.4 ± 335.5^{a,b}	1128.5 ± 193.3^{b}	502.8 ± 42.9	24.3	<0.01
Cardiac sympathovagal balance	0.6 ± 0.1^{b}	0.8 ± 0.2	0.9 ± 0.1	10.0	<0.01
Fasting plasma ghrelin (pmol/l)	301.7 ± 18.9^{a,b}	202.8 ± 15.6^{b}	150.3 ± 7.5	34.2	<0.01
Fasting serum GH (μg/l)	3.7 ± 0.6^{b}	2.4 ± 0.7^{b}	1.2 ± 0.2	17.4	<0.01
Fasting serum leptin (μg/l)	3.6 ± 0.5^{b}	5.2 ± 0.7^{b}	8.6 ± 0.7	25.6	<0.05
Fasting serum amylase (U/l)	136.2 ± 15.5^{a,b}	89.1 ± 5.3	83.7 ± 2.0	17.0	<0.01
Fasting serum potassium (mmol/l)	4.1 ± 0.1	4.2 ± 0.1	4.3 ± 0.1	5.9	0.06
Fasting plasma glucose (pmol/l)	4.5 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	3.6	0.17
Binge-eating and vomiting episodes (times/week)	9.3 ± 0.9^{a,b}	2.1 ± 0.4^{b}	0.0	51.5	<0.01
T scores of depression scale in POMS	70.5 ± 0.7^{a,b}	61.1 ± 2.5^{b}	46.9 ± 1.0	36.3	<0.01

* vs. control group, compared using ANOVA and a post hoc Fisher's test ($P < 0.01$).
$^{a}P < 0.05$ vs. after treatment, $^{b}P < 0.05$ vs. control group, using a chi-square statistic test.

Results

Except for two patients, all participants completed the 16-week treatment without any remarkable side effects. Comparison of physiological characteristics across the study groups is shown in Table 1. The mean levels of plasma ghrelin, cardiac vagal tone and serum GH and amylase in BN patients before treatment were significantly higher than those in the control group ($P < 0.01$). On the contrary, mean levels of BMI, percent body fat, cardiac sympathovagal balance and serum leptin in BN before treatment were significantly lower than those observed in the controls ($P < 0.01$). The elevated levels of plasma ghrelin, cardiac vagal tone and serum amylase of patients with BN before treatment decreased significantly after the 16-week treatment ($P < 0.01$). The leptin levels were not significantly different before and after treatment, although there was a tendency towards an increase after treatment. The levels of BMI, percent body fat, cardiac sympathovagal balance and serum potassium were not significantly different before and after treatment ($P > 0.20$). The levels of ghrelin ($P < 0.01$), cardiac vagal tone ($P < 0.05$), GH ($P < 0.01$) and leptin ($P < 0.01$) in patients after treatment were significantly different versus those observed in the control. Both the frequency of sets of binge-eating and vomiting episodes per week and the *T* scores for the POMS depression scale were significantly improved after treatment ($P < 0.01$).

Discussion

The main findings of the present study were as follows: 1) untreated BN patients had higher fasting ghrelin levels and resting cardiac vagal tone, and lower fasting leptin levels and resting cardiac sympathovagal balance, even though BMI was lower in patients than in controls; 2) the combined SSRI paroxetine treatment with cognitive–behavioral therapy decreased the elevated fasting ghrelin levels and resting cardiac vagal tone, frequency of sets of binge-eating and vomiting episodes and depression scale scores in BN patients despite similar BMI and percent body fat. Our results suggest that both neuroendocrine ghrelin and autonomic nervous vagal functions are dysregulated in BN but improve in parallel with recovery in eating behavior and depression after treatment.

Ghrelin secretion is regulated via the vagal system (Masuda et al., 2000; Asakawa et al., 2001; Date et al., 2002). The dorsal motor nucleus of the vagus projects to the heart and the intestine, and the activity of vagal neurons within this nucleus may coregulate the vagal tone of these organs (Porges, 1995). Since afferent vagal hyperactivity is an important factor in the physiology of BN (Faris et al., 2000), the present results seem to indicate that the increased vagal activity is associated with the increased fasting ghrelin in BN. In addition, our recent studies have shown that a difference in the eating pattern might induce changes in both fasting levels (Tanaka et al., 2003b) and the effects of oral glucose administration on ghrelin as well as insulin in anorexia nervosa (Tanaka et al., 2003c). These findings suggest that habitual abnormal eating behavior may relate to the dysregulation of gastrointestinal hormones via the vagal system. Measuring fasting ghrelin levels and resting cardiac vagal tone may be useful indexes in the treatment of BN patients.

In the present study, the combination of SSRI paroxetine treatment with cognitive–behavioral therapy successfully reduced the frequency of sets of binge-eating and purging episodes and increased cardiac vagal tone, similar to that seen with fluoxetine treatment (Rissanen et al., 1998) and ondansetron, which is a peripherally active antagonist of the serotonin receptor 5-HT3 (Faris et al., 2000). Fluoxetine has been reported to inhibit the serotonin-3-receptor-mediated ionic current (Fan, 1994a) and to facilitate serotonin-3 receptor desensitization (Fan, 1994b). Since vagal neurons at both the peripheral and central levels are endowed with serotonin-3 receptors and agonism at these receptors is conducive to vomiting (Andrews et al., 1990; Knox et al., 1994), the pharmacological effect on serotonin-3 receptors of ondansetron and fluoxetine is thought to be responsible for their beneficial effects in BN. Though paroxetine is not reported to have similar effects on the serotonin-3 receptors, the effect of chronic paroxetine treatment on 5-HT2C/5-HT2B receptor responsivity has been found to be similar to that observed for fluoxetine (Kennett et al., 1994). The 5-HT2C receptor agonist has induced hypophagia and reduced body weight gain in rats (Vickers et al., 2003). Impulsive and depressive symptoms in BN patients have all been associated with abnormalities in brain 5-hydroxytryptamine mechanisms (Jimerson et al., 1990; Steiger et al., 2001). However, it has yet to be determined whether the abnormalities reflect state effects or are a consequence of BN, trait effects in the pathophysiology of BN or a combination of both.

The present results indicate that not only abnormal eating behavior but also depressive symptoms are improved after treatment in BN patients. Improvement of abnormal eating behavior and depressive symptoms occurred in parallel with the recovery of neuroendocrine ghrelin and cardiac vagal function, which was in contrast to that seen for serum GH, amylase or leptin. The combination of SSRI paroxetine with cognitive–behavioral therapy was effective in the treatment of BN, however, attention needs to be paid to discontinuation symptoms that may occur with cessation of paroxetine administration (Judge et al., 2002). Moreover, it may be desirable to continue treatments over a prolonged period of time (Agras et al., 1992; Walsh et al., 1997). We suggest that fasting ghrelin levels and resting cardiac vagal tone may be useful indexes in the assessment of therapeutic efficacy and the clinical condition of BN patients.

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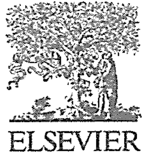
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Ghrelin stimulates growth hormone secretion and food intake in aged rats

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Abstract

Age-related decreases in energy expenditure have been associated with the loss of skeletal muscle and decline of food intake, possibly through a mechanism involving changes of growth hormone (GH) secretion and feeding behavior. Age-related declines of growth hormone secretion and food intake have been termed the somatopause and anorexia of ageing, respectively. Ghrelin, a 28-amino-acid peptide, was isolated from human and rat stomachs as an endogenous ligand of growth hormone secretagogue receptor. Ghrelin stimulates growth hormone release and food intake when peripherally administered to rodents and humans. Here, we investigate the relationship between age-related decline of growth hormone secretion and/or food intake and ghrelin function. Ghrelin (10 nmol/kg body weight) was administered intravenously to male 3-, 12-, 24-and 27-month-old Long-Evans rats, after which growth hormone concentrations and 2 h food intake were measured. An intravenous administration of ghrelin to rats increased food intake in all generations. In addition, to orexigenic effect by ghrelin, intravenous administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration. These findings suggest that the aged rats maintain the reactivity to administered exogenous ghrelin.
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Keywords: Ghrelin; Feeding behavior; Growth hormone secretion; Ageing

1. Introduction

The decline in blood levels of growth hormone (GH) with ageing are commonly referred to as the somatopause (Anawalt and Merriam, 2001; Lamberts et al., 1997). Because GH changes are associated with declines in physical abilities, attempts are often made to save the decline of physical abilities with ageing by GH replacement. However, the relative ratio of risk to benefit in GH replacement requires further discussion. Underlying mechanism of age-related somatopause, therefore, has to be investigated to find an ideal method of intervention.

Ghrelin, a 28-amino-acid peptide, was isolated from human and rat stomachs as an endogenous ligand of growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). Ghrelin stimulates growth hormone release when peripherally or centrally administered to rats and when applied directly to rat primary pituitary cells (Date et al., 2000; Kojima et al., 1999; Toshinai et al., 2006; Wren et al., 2000). Plasma ghrelin levels decline with ageing due to impaired function of the gastric mucosa reducing the thickness of the membrane, the length of the glands, and the number of the endocrine cells in mice (Sandstrom et al., 1999). Previous human studies indicated that stomach ghrelin secretion decreases with ageing (Rigamonti et al., 2002) and that ghrelin-induced GH secretion is reduced in aged subjects compared to younger subjects (Broglio et al., 2003). In contrast to human data, plasma ghrelin concentrations and stomach ghrelin contents in aged rats are significantly higher than in young rats (Englander et al., 2004). In addition, ghrelin-induced GH secretion is higher compared to young rats. However, since these findings were provided from a cross-sectional study, the relationship between age-related dynamics of ghrelin and somatopause remains undefined.

Abbreviations: CCK, cholecystokinin; GH, growth hormone; GHS-R, growth hormone secretagogue receptor; GHRP-6, GH-releasing hexapeptide; IGF-1, insulin-like growth factor-1; ip, intraperitoneal; iv, intravenous; LETO, Long-Evans Tokushima Otsuka

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Anorexia is commonly associated with ageing (MacIntosh et al., 2000; Morley, 2001a) and may be related to age-related decline of plasma ghrelin (Rigamonti et al., 2002). Normal ageing is associated with a decrease in appetite and energy intake, which has been termed the anorexia of ageing (Morley and Thomas, 1999; Morley, 2001b). Generally, after age 70–75 years, the reduction in energy intake exceeds energy expenditure in humans, resulting in weight loss where loss of muscle (sarcopenia) predominates and predisposes older subjects to protein energy malnutrition (Baumgartner et al., 1998; Morley, 2001b). The observed malnutrition and sarcopenia correlates with increased morbidity, and the number of hospitalizations with extended stays (Sullivan, 1998). The causes of the physiological anorexia typified during ageing are unknown; they are probably multifactorial and include a reduction in feeding drive with increased activity of satiety signals. Ghrelin stimulates food intake as well as GH secretion (Asakawa et al., 2001; Lawrence et al., 2002; Nakazato et al., 2001; Shintani et al., 2001; Tschöp et al., 2000; Wren et al., 2000, 2001). Treatment with exogenous ghrelin or ghrelin mimetics may prove beneficial in the anorexia of ageing. To investigate the relationship between age-related decline of GH secretion and food intake and ghrelin function, ghrelin (10 nmol/kg body weight) was administered intravenously to 3-, 12-, 24- and 27-month-old rats, after which GH concentrations and 2 h food intake were measured.

2. Materials and methods

2.1. Animals

Male Long-Evans Tokushima Otsuka (LETO) rats (4-week-old) were obtained from Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All animals were housed individually in plastic cages at constant room temperature (23 C) in a 12 h light (8 AM–8 PM)/12 h dark cycle and were given standard laboratory chow and water *ad libitum*. Experiments were conducted on rats at 3, 12, 24, and 27 months of age ($n = 10$). In this study, anesthesia was carried out by an intraperitoneal (ip) injection of sodium pentobarbital (75 mg/kg body weight) (Abbot Lab., Chicago, IL). Rats were used as follows. Sterilized intravenous (iv) cannulae were implanted into the right jugular vein 1 week before the experiments of feeding and GH response on rats at 3, 12, 24, and 27 months of age. All rats recovered from surgery within 1 week, showing food intake amounts similar to pre-surgery levels and progressive weight gain. These rats were then used in the experiments. Rat ghrelin (Peptide Institute Inc., Osaka, Japan) or saline was administered icv to rats fed *ad libitum*. The 2 h food intake amounts were then measured. This feeding test was performed using a crossover design experiment in which animals were randomized to receive either test substance with a washout period of 2 days between each administration. Two days after the feeding test, ghrelin (10 nmol/kg body weight) was administered iv to these rats which were anesthetized by sodium pentobarbital for the GH response test. After these tests, the iv cannulae were removed from the rats using sterilized devices. To prevent suppuration by infection, we frequently disinfected the rat, and exchanged cages after the operation. Rats were bred in previously described conditions until reaching the age of the following test. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2. Food intake

During 3 days before administration, 24 h food intake amount was measured each day. Ghrelin (10 nmol/kg body weight) or saline was administered iv to rats at 10:00 AM through an iv cannula. The 2 h food intake amount was then measured. Also, relative amount of ghrelin-induced food intake was evaluated by the ratio of ghrelin-induced food intake to average of 24 h food intake amount during the 3 days. All of the rats used in these experiments were satisfactorily acclimated to handling before iv injections.

2.3. GH response

After anesthesia by an ip injection of sodium pentobarbital, ghrelin (10 nmol/kg body weight) was administered iv to rats at 11:30 AM through an iv cannula. Blood samples (60 μl) were obtained from the tail vein, which was cut 15 mm from the tail end at a depth of about 2 mm by knife, at 0, 15, 30 and 60 min after administration. The plasma concentration of GH was determined with a Biotrak Rat GH RIA kit (Amersham, Buckinghamshire, UK).

2.4. Statistic analysis

Data (mean ± S.E.M.) were analyzed by ANOVA (analysis of variance) and the *post hoc* Scheffe-F test. Differences were considered to be significant when the P values were less than 0.05.

3. Results

3.1. Changes of age-related body weight and food intake

Body weight increased gradually in LETO rats from 3- to 24-month of age. The body weight in 27-month-old LETO rats was significantly decreased compared to 24-month-old LETO rats (Fig. 1A). Food intake for 24 h did not change from 3- to 24-month-old LETO rats, while 24 h food intake in 27-month-old LETO rats was significantly decreased compared to 24-month-old LETO rats (Fig. 1B).

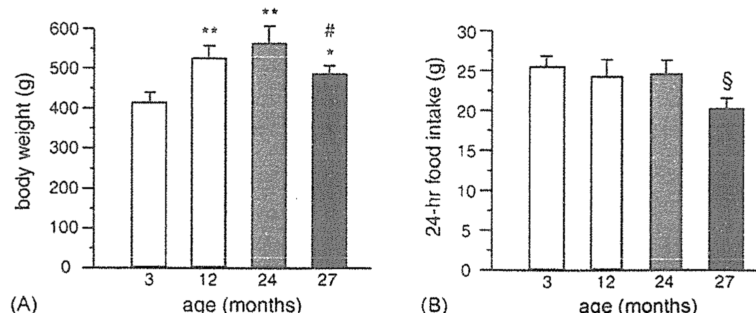

Fig. 1. Changes of body weight (A) and 24 h food intake (B) with ageing. *$P < 0.01$, **$P < 0.001$ vs. 3-month-old rats, #$P < 0.05$ vs. 24-month-old, §$P < 0.01$ vs. 3-, 12-, or 24-month-old rats.

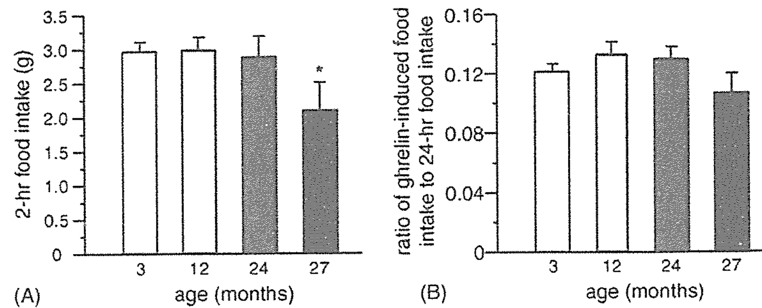

Fig. 2. (A) Effect of iv administration of ghrelin (10 nmol/kg body weight) on 2 h food intake in 3-, 12-, 24- and 27-month-old rats. *$P < 0.01$ vs. 3-, 12-, or 24-month-old rats. (B) No effect of ageing on the ration of ghrelin-induced 2 h food intake to 24 h food intake.

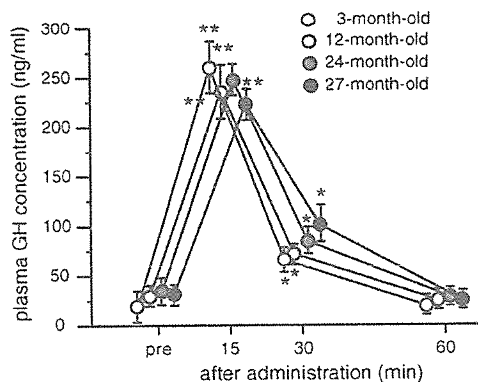

Fig. 3. Effect of iv administration of ghrelin (10 nmol/kg body weight) on the plasma GH concentration in 3-, 12-, 24- and 27-month-old rats. *$P < 0.01$, **$P < 0.001$ vs. pre-administration.

3.2. Changes of age-related ghrelin-induced food intake

We examined the effects of ageing on ghrelin-induced food intake. While an iv administration of saline to LETO rats did not induced food intake in all generations, an iv administration of ghrelin to LETO rats increased food intake in all generations. The amounts of ghrelin-induced food intake in 27-month-old LETO rats were significantly decreased compared to the other generations (Fig. 2A). However, the ratio of ghrelin-induced food intake to 24 h food intake was the same among the generations (Fig. 2B).

3.3. Changes of age-related ghrelin-induced GH secretion

We studied the release of GH in response to peripheral ghrelin administration at all generations of LETO rats. Iv administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration (Fig. 3). The level of ghrelin-induced GH secretion was not different among the generations.

4. Discussion

The circulation level of insulin-like growth factor-1 (IGF-1) is increased by the increase in plasma GH concentration. GH and IGF-1 promote cell survival and proliferation through independent and different pathways (Baixeras et al., 2001). The amplitude of pulsatile GH release from the anterior pituitary gland secretion is attenuated with ageing, and the attenuation of GH release induces decrease in IGF-1 (Ho et al., 1987; Minisola et al., 1993). These age-related reductions are commonly referred to as the somatopause (Anawalt and Merriam, 2001; Lamberts et al., 1997). The somatopause during ageing has been partially explained by the reduction in GH response to peptidyl or nonpeptidyl synthetic ghrelin mimetics, GH-releasing hexapeptide (GHRP-6) or MK-0677, and GH releasing hormone (Abribat et al., 1991; Aloi et al., 1994; Ceda et al., 1986; Chapman et al., 1996; Muccioli et al., 2002; Sonntag et al., 1983; Spik and Sonntag, 1989). The GH responses to acute iv administration of ghrelin in elderly subjects were lower than those in young adult subjects (Broglio et al., 2003). In addition, expression of GHS-R messenger ribonucleic acid is reduced in the aged human hypothalamus, which is consistent with their reduced GH response to ghrelin (Muccioli et al., 2002). Plasma ghrelin concentrations reduce in humans as they age (Rigamonti et al., 2002); therefore, lower ghrelin production in addition to reduced GHS-R levels suggest that somatopause may reflect impairment in the ghrelin signaling pathway. In contrast to humans, stomach ghrelin production and secretion are increased, and GH release in response to exogenous ghrelin is enhanced in aged rats (Englander et al., 2004). Therefore, age-related decline in GH secretion may not be due to a reduction in stomach ghrelin secretion or a stimulatory action on GH release. The present study demonstrated that iv administration of ghrelin increased GH secretion in all LETO rats investigated for 27 months at 15 min after administration. In addition, the levels of GH response to ghrelin were not affected with the months of age in rats. These findings suggest that the aged rats maintain a high reactivity to ghrelin stimulation, and that aged rats secure storage of GH in the anterior pituitary gland.

Longitudinal studies have demonstrated a decline in energy intake with ageing (Hallfrisch et al., 1990; Koehler, 1994). For example, a study involving a three-decade follow-up of 105 male humans aged 27–65 years demonstrated a decrease in daily energy intake of up to 25% (Hallfrisch et al., 1990). A 7-year longitudinal study in subjects aged 64–91 years also demonstrated a decrease in energy intake of 19.3 kcal/d per year in women and 25.1 kcal/d per year in men (Koehler, 1994).

The reduction in energy intake with ageing exceeds energy expenditure, resulting in weight loss involved sarcopenia (Baumgartner et al., 1998; Morley, 2001b). Indeed, the satiating effects of cholecystokinin (CCK), a gastrointestinal-derived anorectic peptide, increased with ageing and fasting and postprandial CCK concentrations are higher in healthy elderly subjects compared to young adults (MacIntosh et al., 1999, 2001). In contrast to age-related increase of CCK function, previous cross-sectional studies indicated that stomach ghrelin secretion and ghrelin-induced GH secretion decreased in aged subjects compared to younger subjects (Broglio et al., 2003; Rigamonti et al., 2002). The efficiency of ghrelin and CCK signal transduction depend on the balance of their respective plasma concentration and/or on interactions between GHS-R and CCK type A receptor (Date et al., 2005). Thus, enhanced effects of CCK and/or reduced effects of ghrelin may contribute to the development of anorexia and in some cases protein malnutrition during ageing. Therefore, ghrelin coupled with its anabolic effects via the GH/IGF-1 axis indicate that rescue of reduced GHS-R activity by treatment with exogenous ghrelin or ghrelin mimetics may contribute to retard the progress of anorexia of ageing. We indicate that iv administration of ghrelin increases food intake in all generations and that the ratio of ghrelin-induced food intake to 24 h food intake was the same among the generations. These results suggest that peripheral administration of ghrelin may prevent age-dependent decline in energy intake in animals.

Recent studies demonstrated that circulating ghrelin bound to the membranes of cardiomyocytes, adipocytes, and osteocytes dependently or independently of the GHS-R (Baldanzi et al., 2004; Bedendi et al., 2003; Delhanty et al., 2006). Ghrelin functions as an anti-catabolic agent in peripheral tissues, involving adipogenesis, osteogenesis, and cell proliferation (Baldanzi et al., 2004; Bedendi et al., 2003; Delhanty et al., 2006). Therefore, ageing process represented by catabolic-anabolic imbalance in peripheral tissues may increase ghrelin utilization to maintain cell functions. The present study indicated the possibility of suppressing the age-related decline of GH secretion and food intake by ghrelin. Further studies will be necessary to clarify whether a chronic administration of ghrelin prevents age-related regression involved somatopause, sarcopenia, and anorexia.

In conclusion, our results indicate that peripheral administration of ghrelin increases GH secretion and food intake in all generations. Somatopause and anorexia of ageing are associated with declines in physical abilities. Therefore, ghrelin replacement may improve physical abilities to stimulate GH secretion and feeding in aged animals. The present study will provide novel insights into the physiological function of ghrelin in ageing process.

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Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay

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ABSTRACT Two forms of ghrelin, acylated and desacyl, circulate in plasma. Although acylation is thought to be essential for ghrelin biological activities, recent studies have suggested that desacyl ghrelin may also possess biological activity. A new commercial ELISA system has now enabled us to measure plasma levels of each of these two ghrelin forms separately. This assay system directly measures levels using small amounts of plasma. To evaluate the utility of this assay system, we measured the plasma levels of the two forms of ghrelin in healthy volunteers. Although acylated ghrelin levels were equivalent to those measured previously by RIA, desacyl ghrelin levels were lower than those expected from the total ghrelin levels previously determined by RIA. The ratios of acylated to desacyl ghrelin significantly correlated with previously determined acylated, but not desacyl, ghrelin levels. After BMI adjustment, the levels of acylated, but not desacyl, ghrelin plasma levels were higher in female subjects than those in males. Several metabolic and hormonal parameters significantly correlated with either plasma acylated or desacyl ghrelin levels. These findings indicate that separate measurements of the two ghrelin form levels may provide valuable information on their structure, gender differences, and physiological implications.

Ghrelin, a 28 amino acid peptide, exhibits a variety of biological activities, including the stimulation of both GH release and food intake (1-3). Plasma ghrelin levels are elevated after fasting and reduced following feeding (4, 5). Plasma ghrelin concentrations negatively correlate with increasing body mass index (BMI); levels are decreased in obese subjects and increased in anorexia nervosa patients (5-8). In addition, low ghrelin plasma levels are associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes (9), prompting the hypothesis that circulating ghrelin levels are related to appetite, energy homeostasis, and glucose metabolism.

A portion of ghrelin possesses a unique fatty acid modification, an *n*-octanoylation, at Ser 3 (1). Of the two circulating ghrelin forms, acylated and unacylated (desacyl), the acylated form is thought to be essential for ghrelin biological activity. Desacyl ghrelin, however, has recently been reported to influence cell proliferation and adipogenesis (10-13). To better understand the roles of the individual ghrelin forms, it will be important to measure plasma levels of both acylated and desacyl ghrelin separately.

Previous measurements of circulating ghrelin levels in humans, however, have been performed using an antiserum which recognizes both forms, providing a measure of total ghrelin levels. Commercial radioimmunoassays (RIAs) from both Phoenix Pharmaceuticals (cat. No. RK-031-30) and Linco Research (cat. No. GHRT-89HK) can only measure total ghrelin levels (14). Although our RIA assays, N-RIA and C-RIA, can measure acylated ghrelin and total ghrelin, respectively (15), our determinations of ghrelin concentrations by N-RIA were limited to patients with renal diseases (16) and anorexia nervosa (17). Although an assay analyzing acylated ghrelin levels is now commercially available from Linco (cat. No. GHRA-88HK), few studies examining ghrelin levels in human plasma samples using this assay have yet been reported (18). The recent availability of a new enzyme-linked immunosorbent assays (ELISAs) that can measure both acylated and desacyl forms of ghrelin from Mitsubishi Kagaku Iatron, Inc. (Tokyo, Japan) provides the first assay that can specifically measure the desacyl form oghrelin in small quantities of plasma (50 μl/well). In addition, this assay system does not require the complicated and time-consuming purification of ghrelin using a Sep-Pak C_{18} cartridge. In this study, we measured the plasma levels of acylated and desacyl ghrelin in healthy volunteers using these new ELISA kits. To explore the physiological implications of these two ghrelin forms, we examined the gender differences in plasma ghrelin levels and their correlations with hormonal and metabolic parameters.

Materials and Methods

Subjects

Sixteen male and 20 female volunteers between 21 and 61 years of age participated in our study. All of the subjects are Japanese and did not suffer from any known medical conditions. None of the subjects were currently taking medication. The study protocol was approved by the ethics committee on human research at Kyoto University Graduate School of Medicine and Kansai Medical School. Written informed consent was obtained from all subjects prior to enrollment.

Measurement of the concentrations of plasma ghrelin and glucose and serum nonesterified fatty acid (NEFA), GH, IGF-1, insulin, and leptin

Blood samples for hormone and glucose analyses were drawn

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from a forearm vein in the morning after overnight fasting. Plasma samples were prepared as previously described (1). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan: 500 kallikrein inactivator U/ml), centrifuged at 4°C. To the separated plasma was immediately added 1N HCl (10% volume of plasma volume). The acylated and unacylated forms of ghrelin were measured using two commercially available ELISA kits, Active Ghrelin ELISA Kit and Desacyl-Ghrelin ELISA Kit, respectively, according to the manufacturer's protocol (Mitsubishi Kagaku Iatron, Inc.) (19, 20). The minimal detection limits of acylated and desacyl ghrelin in this assay system were 2.5 and 12.5 fmol/ml, respectively. The intra- and interassay coefficiencies of variation were 6.5% and 9.8%, respectively, for acylated ghrelin and 3.7% and 8.1%, respectively, for desacyl ghrelin. Plasma glucose was measured using the glucose oxidase method. NEFA was measured enzymatically. Serum GH, IGF-1, and insulin were measured by immunoradiometric assay (IRMA), while serum leptin was measured by radioimmunoassay (RIA) (Mitsubishi Kagaku Bio-Clinical Laboratories, inc., Tokyo, Japan). Insulin resistance was calculated according to the homeostatis model of assessment-insulin resistance (HOMA-IR), calculated as insulin (μU/ml) x blood glucose (mmol/l) /22.5 (21).

Statistical analysis

Data are expressed as the mean ± SD. Statistical evaluations were performed using Student *t*-test, Pearson's rank correlation, and multiple-regression analyses as appropriate. P values less than 0.05 was considered statistically significant.

Results

Plasma ghrelin concentrations in volunteers

We examined the anthropometric, hormonal, and metabolic parameters in volunteers (Table 1). The mean levels of plasma acylated and desacyl ghrelin in the 36 volunteers were 15.9 ± 9.4 and 66.1 ± 45.3 fmol/ml, respectively. There was an excellent correlation between acylated and desacyl ghrelin levels (r = 0.840, p < 0.0001). The ratio of acylated to desacyl ghrelin (A/D ratio) and the ratio of acylated to[acylated + desacyl] ghrelin levels (A/[A + D] ratio) were 24.9 ± 8.4% and 19.6 ± 5.4 %, respectively (Table 1). The levels of acylated ghrelin correlated significantly with the A/D and A/[A + D] ratios (r = 0.371, p < 0.05), while desacyl ghrelin did not (r = -0.097, p = 0.577).

Plasma ghrelin levels in female subjects were significantly higher than those in male subjects for both acylated (male, 10.9 ± 6.1; female, 19.9 ± 9.8) and desacyl ghrelin (male, 49.1 ± 23.5; female, 79.8 ± 53.9), giving final plasma concentrations 1.8- and 1.6-fold higher in females for acylated and desacyl ghrelin, respectively. BMI, GH, glucose, leptin, and NEFA levels, however, also differed significantly between the genders. As BMI is known to correlate negatively with plasma ghrelin levels (5, 7, 8, 22, 23), we performed a BMI adjustment by multiple regression analysis. The significant gender difference persisted for plasma levels of acylated (p < 0.05), but not desacyl (p = 0.201), ghrelin levels.

Correlations of ghrelin concentrations with various parameters in control subjects

BMI, serum insulin levels, plasma glucose levels, and HOMA-IR negatively correlated with plasma levels of both acylated and desacyl ghrelin, while GH exhibited a positive correlation (Table 2). Age, IGF-1, and leptin levels did not correlate with either ghrelin form.

Discussion

The mean levels of plasma acylated ghrelin in 36 volunteers, 15.9 ± 9.4 fmol/ml, determined using new commercial ELISAs, were comparable with those previously determined for patients with normal renal function (14.7 ± 5.8 fmol/ml) (16) and healthy women (21.2 ± 3.1 fmol/ml) (17) by RIA. In contrast, the mean levels of plasma desacyl ghrelin, 66.1 ± 45.3 fmol/ml, were lower than expected from the total ghrelin levels measured by RIA (145.0 ± 33.5 to 223.4 ± 22.7 fmol/ml (5, 23, 24). The ratio of acylated to [acylated + desacyl] ghrelin (A/[A + D] ratio), 19.6 ± 5.4%, determined here was also lower than that of acylated to total ghrelin, 8.3 ±

Table 1. Plasma ghrelin concentrations and various parameters in healthy subjects

Parameters	All (n = 36)	Male (n = 16)	Female (n = 20)	Male vs Female (P value in *t*-test*)
Age (yr)	33.5 ± 9.0	34.7 ± 7.1	32.6 ± 10.3	0.486
BMI (kg/m^2)	21.7 ± 2.8	23.4 ± 3.0	20.3 ± 1.9	**0.001**
GH (ng/ml)[a]	4.10 ± 6.38	0.29 ± 0.24	7.15 ± 7.27	**0.001**
IGF-1 (ng/ml)	263.1 ± 71.0	258.25 ± 57.8	266.9 ± 81.4	0.722
Insulin (μU/ml)	7.1 ± 4.3	7.95 ± 5.5	6.5 ± 3.0	0.311
Glucose (mg/dl)	88.4 ± 6.6	92.4 ± 4.9	85.3 ± 6.1	**0.001**
HOMA-IR	1.6 ± 1.0	1.8 ± 1.3	1.4 ± 0.7	0.180
Leptin (ng/ml)	7.2 ± 5.2	5.4 ± 2.8	8.7 ± 6.2	0.054
NEFA (mEq/l)	0.43 ± 0.16	0.37 ± 0.14	0.48 ± 0.16	**0.030**
active ghrelin (fmol /ml)	15.9 ± 9.4	10.9 ± 6.1	19.9 ± 9.8	**0.003**
des-acyl ghrelin (fmol /ml)	66.1 ± 45.3	49.1 ± 23.5	79.8 ± 53.9	**0.041**
A/[A + D] ratio	19.6 ± 5.9	18.0 ± 5.6	20.8 ± 5.1	0.129
A/D ratio	24.9 ± 8.4	22.6 ± 8.8	26.8 ± 7.8	0.139

*: p < 0.05. Bold values: P < 0.05.

Systeme International (SI) units for GH, micrograms per liter (conversion factor, 1.0); for IGF-I to nanomoles per liter (0.131); for glucose, millimoles per liter (0.05551); for insulin, picomoles per liter (6.945); for leptin, nanomoles per liter (0.08).

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Table 2. Correlations between plasma ghrelin concentrations and various parameters in healthy subjects

	All (n = 36)		Male (n = 16)		Female (n = 20)	
	r	p	r	p	r	p
Active ghrelin						
Age	-0.256	0.132	-0.219	0.421	-0.232	0.331
BMI	-0.387	**0.019**	-0.195	0.476	-0.193	0.421
GH	0.567	**0.0002**	0.402	0.125	0.470	**0.036**
IGF-1	0.085	0.624	-0.031	0.910	0.085	0.624
Insulin	-0.370	**0.026**	-0.314	0.242	-0.442	0.050
Glucose	-0.535	**0.0006**	-0.175	0.525	-0.450	**0.046**
HOMA-IR	-0.401	**0.015**	-0.327	0.221	-0.463	**0.039**
Leptin	-0.053	0.760	-0.065	0.814	-0.053	0.760
NEFA	0.251	0.140	-0.082	0.766	0.171	0.476
Desacyl ghrelin						
Age	-0.178	0.302	-0.112	0.684	-0.155	0.519
BMI	-0.364	**0.028**	-0.189	0.490	-0.324	0.224
GH	0.446	**0.0058**	0.364	0.169	0.337	0.148
IGF-1	0.149	0.390	0.104	0.706	0.149	0.390
Insulin	-0.381	**0.021**	-0.348	0.191	-0.488	**0.028**
Glucose	-0.508	**0.0013**	-0.090	0.744	-0.495	**0.025**
HOMA-IR	-0.398	**0.015**	-0.350	0.188	-0.504	**0.022**
Leptin	-0.076	0.663	-0.155	0.572	-0.076	0.663
NEFA	0.174	0.312	0.069	0.803	0.044	0.857

r: correlation coefficient. Bold values: $P < 0.05$

2.0 to 9.4 ± 0.8%, previously determined by RIA (16, 24). As RIA measuring total ghrelin is measured using an antiserum against the C-terminal region of ghrelin (human ghrelin-(13-28)), these findings suggest that a fragmented form of ghrelin lacking the N-terminal region may naturally exist in human plasma or may be artificially produced during the RIA procedure. If so, then approximately 40-60% of the total ghrelin measured by RIA is likely fragmented. Alternatively, the differences in these measurements may result from the use of different antibodies in the ELISA and RIA systems. The measured values obtained with Linco total ghrelin RIA are approximately 10-fold higher than those obtained with Phoenix RIA (14). Desacyl Ghrelin ELISA-kit gave similar values to Phoenix RIA and our C-RIA, but not Linco total ghrelin RIA. In addition, the values obtained with Active Ghrelin ELISA-kit were similar to those obtained with our N-RIA, but were approximately one-order lower than those with Linco active ghrelin RIA (18). Thus, only Linco's kits give approximately 10-fold higher values. We agree that an adjustment to one reference standard will be required to solve this discrepancy (14).

Although there is an excellent correlation between acylated and desacyl ghrelin levels, the A/D and A/[A + D] ratios significantly correlated with acylated, but not desacyl, ghrelin quantities. This finding suggests that the activity of acylation enzyme is either increased in hyperghrelinemia or decreased in hypoghrelinemia. Although this result is difficult to interpret from a physiological viewpoint, a similar phenomenon is observed in T3 thyrotoxicosis. Frequently occurring in Graves' disease, type I deiodinase activity, which catalyzes the formation of T3 (active form of thyroid hormone) from T4 (inactive form of thyroid hormone), is increased in hyperthyroidism.

Plasma levels of both acylated and desacyl ghrelin in female subjects were significantly higher than those in male subjects. The significant gender difference in plasma acylated, but not in desacyl, ghrelin levels persisted even when the comparison was normalized to subject BMI. This finding agrees with a report from Chan *et al.*, who demonstrated significantly higher total ghrelin levels in females. This result, however, was no longer significant after normalization to BMI (25). Higher GH levels in females may be partially explained by the elevated acylated ghrelin levels. Although glucose levels were all within normal limits, they are slightly higher in females than in males, requiring the careful interpretation of these results.

By correlation analysis, BMI, serum insulin levels, and HOMA-IR negatively correlated with plasma levels of both acylated and desacyl ghrelin. These results are in line with previous reports of a negative correlation of total ghrelin levels in plasma with BMI (5-8, 23), insulin (6, 9, 26), and insulin resistance (9, 26). Here, we confirmed that this correlation holds true for acylated ghrelin levels as well. GH correlated positively with both acylated and desacyl ghrelin levels. Although we could not identify a correlation with IGF-1 levels, the positive correlation of total ghrelin levels in plasma with serum GH levels has been previously reported (16). The stronger correlation with GH of acylated ghrelin, rather than desacyl ghrelin, suggests the physiological role of ghrelin in GH regulation.

In summary, we measured plasma levels of both acylated and desacyl ghrelin in healthy subjects using novel direct ELISA systems. The comparison of these values to those obtained in our previous RIA systems suggests the presence of ghrelin peptide fragments in plasma. Although acylated ghrelin levels in plasma correlated well with desacyl ghrelin, A/D and A/[A + D] ratios could only be correlated to acylated ghrelin levels. The levels of acylated, but not desacyl, ghrelin in plasma was higher in female volunteers than in males after

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BMI adjustment. BMI, serum insulin levels, and HOMA-IR negatively correlated with both plasma acylated and desacyl ghrelin levels. Serum GH levels correlated closely with plasma acylated, rather than desacyl, ghrelin levels. These findings indicate that the separate measurement of the two forms of plasma ghrelin is useful; plasma acylated ghrelin levels may better reflect the physiological states or alterations in patients more than measurement of plasma desacyl or total ghrelin levels.

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Transgenic Mice Overexpressing Des-Acyl Ghrelin Show Small Phenotype

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Ghrelin, a 28-amino acid acylated peptide, displays strong GH-releasing activity in concert with GHRH. The fatty acid modification of ghrelin is essential for the actions, and des-acyl ghrelin, which lacks the modification, has been assumed to be devoid of biological effects. Some recent reports, however, indicate that des-acyl ghrelin has effects on cell proliferation and survival. In the present study, we generated two lines of transgenic mice bearing the preproghrelin gene under the control of chicken β-actin promoter. Transgenic mice overexpressed des-acyl ghrelin in a wide variety of tissues, and plasma des-acyl ghrelin levels reached 10- and 44-fold of those in control mice. They exhibited lower body weights and shorter nose-to-anus lengths, compared with control mice. The serum GH levels tended to be lower, and the serum IGF-I levels were significantly lower in both male and female transgenic mice than control mice. The responses of GH to administered GHRH were normal, whereas those to administered ghrelin were reduced, especially in female transgenic mice, compared with control mice. These data suggest that overexpressed des-acyl ghrelin may modulate the GH-IGF-I axis and result in small phenotype in transgenic mice. (*Endocrinology* 146: 355–364, 2005)

GHRELIN, AN ACYLATED peptide of 28 amino acids, was identified as an endogenous ligand for the GH secretagogue (GHS) receptor (GHS-R) (1). The major site of production of ghrelin is the stomach and it is also expressed in the hypothalamus (2–5). Plasma ghrelin levels are regulated by acute feeding states. They rise by fasting and are rapidly suppressed by feeding (3, 6–8). Secretion of ghrelin is also regulated by chronic feeding states. Plasma ghrelin levels are elevated in patients with anorexia nervosa and food-restricted animals and are reduced in obese subjects (3, 6–10). These data suggest the possible involvement of ghrelin in energy homeostasis. In fact, ghrelin stimulates food intake in animals and humans and exhibits anticachectic effect in cancer-bearing mice (8, 11–13).

Exogenously administered ghrelin strongly stimulates GH release in a clear dose-dependent manner *in vivo* (1, 2, 14–16). The site of ghrelin action on GH release is not well known to date. The GHS-R is reported to be expressed in the pituitary as well as hypothalamus (17–19). Previous studies indicate that ghrelin binds to membranes from the pituitary and stimulates GH release from cultured pituitary cells (1, 20), suggesting that the pituitary is one of the sites of ghrelin actions. The stimulatory effect of GHSs and ghrelin on GH secretion, however, is more prominent *in vivo* than *in vitro*, and intact GHRH signaling is essential for the effect (1, 21). Hexarelin, one of the potent GHSs, cannot efficiently stimulate GH release in patients with GHRH receptor deficiency (22). Moreover, as we demonstrated, ghrelin has a synergistic action with GHRH. Even a low dose of ghrelin can highly augment GH release by GHRH (23). These data indicate a critical role of the hypothalamus in the stimulatory effect of ghrelin on GH secretion. The strong potency of ghrelin suggests its role as a physiological regulator of GH secretion (1, 2, 14–16). The issue, however, is currently controversial. One recent study (24), using a GHS antagonist, revealed that circulating ghrelin in peripheral blood may not play a role in generating pulsatile GH secretion. Moreover, deletion of ghrelin impairs neither growth nor appetite, indicating that ghrelin is not critically required for GH secretion (25). Another study (26), however, demonstrated that the attenuation of the GHS-R expression *in vivo* results in reduction in food intake and growth, suggesting a physiological role of the ghrelin-GHS-R system in the secretory regulation of GH.

The acylation of ghrelin is assumed to be essential for its actions (1). Des-acyl ghrelin, which lacks the fatty acid modification and circulates at 10-fold higher concentration than acylated ghrelin (1, 3, 27), is devoid of any endocrine activities including GH release, based on previous studies (1, 28). Recent studies (29, 30), however, indicated that des-acyl ghrelin may share with acylated ghrelin the modulation of neoplastic cell proliferation and cardiovascular cell survival *in vitro*. Moreover, one study shows that des-acyl ghrelin may offset the inhibitory effect of acylated ghrelin on insulin secretion (28). Although previous studies indicated that several tissues and cell lines produce des-acyl and/or acylated ghrelin (3, 27, 31, 32), the mechanism by which ghrelin is acylated is also unknown to date.

In the present study, we generated transgenic mice bearing the preproghrelin gene under the control of a cytomegalovirus immediate early enhancer and a modified chicken β-

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Abbreviations: BMI, Body mass index; GHS, GH secretagogue; GHS-R, GHS receptor.

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actin promoter, designated CAG promoter (33, 34). This promoter sequence has been demonstrated to have high activity in cultured cells and transgenic mice (33, 34). Transgenic mice in the present study overexpressed des-acyl ghrelin in plasma and a wide variety of tissues and showed small phenotype. Here we show that des-acyl ghrelin may modulate endogenous ghrelin action and alter the GH-IGF-I axis in transgenic mice.

Materials and Methods

All procedures in animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. The procedures were performed in accordance with the principle and guidelines established by the committee.

Plasmid construction and generation of transgenic mice

The full-length mouse preproghrelin cDNA (1) and the pCAGGS expression vector including the CAG promoter (34) were kindly donated by Professor Masayasu Kojima (Division of Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Japan) and Professor Jun-ichi Miyazaki (Department of Nutrition and Physiological Chemistry, Osaka University School of Medicine, Osaka, Japan), respectively. Plasmid pCAGGS-ghrelin was constructed by inserting the mouse preproghrelin cDNA into the unique *Eco*RI site between the CAG promoter and 3'-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. The DNA fragment was excised from its plasmid by digestion with *Sal*I and *Hind*III and then purified and microinjected into the pronuclei of fertilized eggs obtained from BDF1 female mice (Charles River Japan, Yokohama, Japan) as reported previously (35). Founder transgenic mice were identified by PCR analysis and bred with C57BL/6 mice (Japan CLEA, Osaka, Japan). Mice were housed in air-conditioned animal quarters, with the lights on between 0800 and 2000 h and were given standard rat chow (CE-2, 352 kcal per 100 g, Japan CLEA) and water *ad libitum*.

Measurement of total and acylated ghrelin levels in tissue samples

Tissues such as the stomach, cerebrum, heart, and kidney were removed from 8-wk-old mice under anesthesia with diethyl ether. Each sample was diced and boiled for 7 min in a 5-fold volume of water. The solution was adjusted to 1.0 M acetic acid and 20 mM hydrogen chloride after boiling, and the tissue was homogenized. The supernatant was obtained after centrifugation at 10,000 rpm for 30 min. Tissue ghrelin levels were measured using two kinds of RIAs, C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin as reported previously (9, 27). C-RIA and N-RIA recognize total (acylated plus des-acyl ghrelin) and acylated ghrelin, respectively (9, 27).

Measurement of plasma total and acylated ghrelin levels

Blood samples were collected from the inferior vena cava of mice under anesthesia with diethyl ether. The samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan; 1000 kallikrein inactivator U/ml) and centrifuged at 4 C. For N-RIA, hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after the separation of plasma. Plasma ghrelin was measured as reported previously (1, 3, 27). Briefly, the samples were subjected to a Sep-Pak C18 cartridge and C-RIA and N-RIA were carried out.

Measurement of body weights and lengths, organ weights, and daily food intake

Body weights of control and transgenic mice were measured weekly, beginning at 3 wk of age. Body lengths of 8- and 52-wk-old mice were measured by manual immobilization and extension of mice to the nose-to-anus lengths, always by the same individual. Body mass indexes (BMIs = weight/(nose-to-anus lengths)^2) were calculated in 8- and 52-wk-old control and transgenic mice (36, 37). Organs such as the pituitary, stomach, cerebrum, heart, liver, kidney, spleen, pancreas, and epididymal fat were removed from 8-wk-old mice under anesthesia with diethyl ether and weighed. Daily food intake was monitored for 3 wk, beginning at 5 wk of age.

Measurement of blood glucose, serum total protein, total cholesterol, and hormones

To examine the nutritional conditions, blood glucose and serum total protein and total cholesterol levels were measured. Eight-week-old control and transgenic mice were used. Four hundred microliters of blood samples were collected from the tail vein of mice for blood glucose levels at 1000 h after 12 h fasting. Then the mice were anesthetized with diethyl ether, and 400 μl of blood samples were collected from the inferior vena cava for serum total protein, total cholesterol, and hormone levels. Blood glucose, serum total protein, and total cholesterol levels were measured by the glucose oxidase method with a reflectance glucometer (One Touch II; Lifescan, Milpitas, CA), BCA protein assay reagent kit (Pierce, Rockford, IL), and Amplex red cholesterol assay kit (Molecular Probes, Eugene, OR), respectively. Serum GH and IGF-I levels were measured with EIA kits (SPI-BIO, Bonde, France, and Diagnostic Systems Laboratories Inc., Webster, TX, respectively). Serum insulin and plasma ACTH levels were measured with EIA kits (Morinaga, Tokyo, Japan) and ACTH-RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), respectively. Serum TSH, LH, and FSH levels were measured with EIA kits (Amersham Biosciences, Buckinghamshire, UK).

Effects of GHRH and ghrelin on serum GH levels

Human GHRH and rat ghrelin were purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan) and Peptide Institute, Inc. (Osaka, Japan), respectively. Male and female 8-wk-old control and Tg 10–1 mice were used under no anesthesia. Control and transgenic mice were housed in the same cage and tested on the same day. Forty mice were divided into five groups for blood sampling. Eight mice in the same group were used for each blood sampling. Control and transgenic mice were iv injected with human GHRH (60 μg/kg) or rat ghrelin (40 μg/kg). Four hundred microliters of blood samples were collected from the inferior vena cava of mice 0, 10, 20, 30, and 60 min after the injection. Serum GH levels were measured with an EIA kit (SPI-BIO).

Real-time PCR analysis of preproghrelin, GH, GHRH, somatostatin, and GHS-R mRNAs

Total RNAs from tissues, such as the stomach, small intestine, cerebrum, hypothalamus, pituitary, liver, kidney, lung, heart, and skeletal muscle, were extracted using the acid guanidinium thiocyanate-phenol-chloroform method (38). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript II RT (Life Technologies, Inc., St. Louis, MO) with random hexamers according to the manufacturer's instructions. Taqman-PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using VIC-labeled fluorogenic probes specific for preproghrelin, GH, GHRH, somatostatin, or GHS-R transcript, or the internal standard glyceraldehyde-3-phosphate dehydrogenase. Oligo primers and probes (Table 1) were chosen using the Primer Express software (Applied Biosystems). The PCR was performed using Taqman Universal PCR Mastermix (Applied Biosystems) to which primers and probes were added (final concentrations 400 and 200 nM, respectively). All samples were run in triplicate in 96-well plates in the ABI Prism 7700 sequence detector according to the manufacturer's standard protocol. For the primer sets, serial dilutions were conducted with different cDNA preparations to confirm the kinetics of the PCR. There was no significant difference in glyceraldehyde-3-phosphate dehydrogenase mRNA levels among experimental groups.

Effects of continuous infusion of des-acyl ghrelin on the GH-IGF-I axis and body weights

Rat des-acyl ghrelin was purchased from Peptide Institute, Inc. Des-acyl ghrelin was dissolved in saline at a concentration of 700 μg/ml and stored in osmotic minipumps (DURECT Corp., Cupertino, CA). The

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TABLE 1. Primer and probe sequences for real-time PCR analysis for preproghrelin, GH, GHRH, somatostatin, and GHS-R mRNAs

	Primer and probe sequences
Preproghrelin	
Forward	5'-GCATGCTCTGGATGGACATG-3'
Reverse	5'-TGGTGGCTTCTTGGATTCCT-3'
Probe	5'-AGCCCAGAGCACCAGAAAGCCCA-3'
GH	
Forward	5'-AAGAGTTCGAGCGTGCCTACA-3'
Reverse	5'-GAAGCAATTCCATGTCGGTTC-3'
Probe	5'-CCATTCAGAATGCCCAGGCTGCTTTC-3'
GHRH	
Forward	5'-AGGATGCAGCGACACGTAGA-3'
Reverse	5'-TCTCCCCTTGCTTGTTCATGA-3'
Probe	5'-CCACCAACTACAGGAAACTCCTGAGCCA-3'
Somatostatin	
Forward	5'-AGCTGAGCAGGACGAGATGAG-3'
Reverse	5'-ACAGGATGTGAATGTCTTCCAGAA-3'
Probe	5'-CGAACCCAGCAATGGCACCCC-3'
GHS-R	
Forward	5'-CACCAACCTCTACCTATCCAGCAT-3'
Reverse	5'-CTGACAAACTGGAAGAGTTTGCA-3'
Probe	5'-TAAGATCTGCTCATCTTAATGTGCATG-3'

minipumps were implanted into the peritoneum. Des-acyl ghrelin or saline was infused continuously through the minipumps into 4-wk-old C57/BL6 mice (Japan CLEA) for 10 d. The minipumps were continuously delivering saline or 250 μg/kg·d of des-acyl ghrelin for 10 d at a speed of 0.22 μl/h. Body weights were measured daily for 10 d. Four hundred microliters of blood samples for the measurement of serum GH and IGF-1 levels were collected from the inferior vena cava of mice under anesthesia with diethyl ether 10 d after the implantation.

Hematoxylin eosin and immunohistochemical staining for total ghrelin, acylated ghrelin, and GH of the pituitary

The pituitaries were removed from male 8-wk-old mice under anesthesia with diethyl ether and fixed with 4% paraformaldehyde and 0.2% picric acid and embedded in paraffin. The tissues were cut in 3-μm-thick slices. Samples were subjected to immunohistochemical staining for total and acylated ghrelin as well as hematoxylin eosin staining. After pretreatment with 0.3% hydrogen peroxide and incubation with normal goat serum, all slices were incubated overnight at 4 C with ghrelin(13–28) antiserum recognizing total (des-acyl plus acylated) ghrelin (final dilution, 1:5000), antighrelin(1–11) antiserum specifically recognizing acylated ghrelin (final dilution, 1:5000), or anti-GH antiserum (Biogenesis, Poole, UK) (final dilution, 1:200). All of the sections were stained by the avidin-biotin complex method and counterstained with hematoxylin as reported previously (39).

Statistical analysis

Results are expressed as the mean ± SEM. ANOVA followed by the t test was used to assess differences between control and transgenic mice. $P < 0.05$ was considered to be statistically significant.

Results

Generation of transgenic mice and preproghrelin mRNA levels

Two lines of transgenic mice with six (Tg 10–1) and 12 (Tg 9–2) copy numbers were identified by PCR and Southern blot analysis. Preproghrelin mRNAs were detected only in the stomach, small intestine, lung, pituitary, and hypothalamus of control mice, and the amounts were 100, 4, 2.1, 1.5, and 0.5 in arbitrary units (AU), respectively (Fig. 1). On the other hand, they were detected in all tissues examined in Tg 9–2 and Tg 10–1 mice, and the amounts in the stomach of Tg 9–2 and Tg 10–1 mice reached 1100 and 5200 AU, respectively (Fig. 1). Preproghrelin mRNA levels in other tissues of Tg 9–2 and Tg 10–1 mice also exceeded those of control mice.

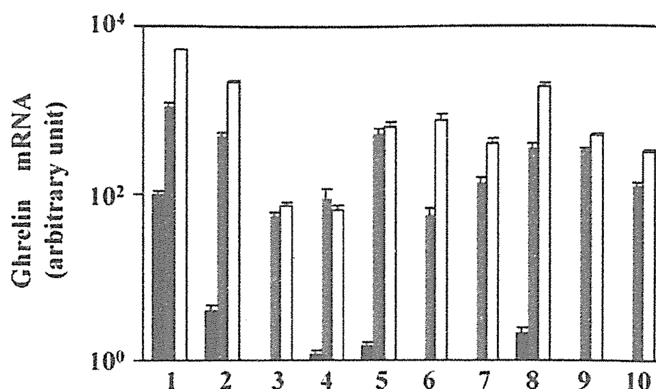

FIG. 1. Preproghrelin mRNA levels in the tissues of control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis. Lanes 1, stomach; 2, small intestine; 3, cerebrum; 4, hypothalamus; 5, pituitary; 6, liver; 7, kidney; 8, lung; 9, heart; and 10, skeletal muscle.

Total and acylated ghrelin levels in tissues and plasma

Eight-week-old control, Tg 9–2, and Tg 10–1 mice were used (Table 2). Although high total ghrelin levels were detected in the stomach, only very low levels were detected in other tissues of control mice. Tg 9–2 and Tg 10–1 mice showed significantly higher total ghrelin levels in the stomach than control mice ($P < 0.01$ for each). Tg 9–2 and Tg 10–1 mice also showed total ghrelin levels in all of the other tissues significantly higher than control mice. High levels of acylated ghrelin were also detected in the stomach of control, Tg 9–2, and Tg 10–1 mice. There was, however, no significant difference between control and Tg 9–2 mice and between control and Tg 10–1 mice. Only very low acylated ghrelin levels if any were detected in other tissues of control, Tg 9–2, and Tg 10–1 mice. Plasma total ghrelin levels in control, Tg 9–2, and Tg 10–1 mice were 1104.5 ± 94.4, 11230.6 ± 1147.1, and 48565.5 ± 9291.5 fmol/ml, respectively. Those in Tg 9–2 and Tg 10–1 mice were significantly higher than those in control mice ($P < 0.01$ for each). Plasma acylated ghrelin levels in control, Tg 9–2, and Tg 10–1 mice were 83.7 ± 11.9, 79.7 ± 10.1, and 86.3 ± 21.1 fmol/ml, respectively. The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant.

Body weights and lengths, relative organ weights, and BMIs

Body weights of control, Tg 9–2, and Tg 10–1 mice are shown in Table 3 and Fig. 2A. Male Tg 9–2 and Tg 10–1 mice were significantly lighter in the body weight than control mice ($P < 0.05$ and $P < 0.01$, respectively). Female Tg 10–1 mice were also significantly lighter than control mice ($P < 0.01$). The difference between female control and Tg 9–2 mice was not significant. Fifteen-week-old male and female Tg 10–1 and male Tg 9–2 mice were still significantly lighter than control mice ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Body lengths (nose-to-anus lengths) of control and

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TABLE 2. Total and acylated ghrelin levels in plasma and tissues of 8-wk-old control and transgenic mice (n = 8/group)

	Control	Tg 9-2	Tg 10-1
Total ghrelin			
Plasma (fmol/ml)	1104.5 ± 94.4	11230.6 ± 1147.1[a]	48565.5 ± 9291.5[a]
Tissues (fmol/mg)			
Stomach	2191.9 ± 340.9	2860.8 ± 587.3[a]	5430.6 ± 626.1[a]
Cerebrum	0.8 ± 0.2	34.3 ± 4.2[a]	110.9 ± 41.0[a]
Heart	1.4 ± 0.2	27.6 ± 5.6[a]	30.2 ± 9.3[a]
Kidney	1.9 ± 0.1	43.5 ± 5.9[a]	68.3 ± 10.5[a]
Acylated ghrelin			
Plasma (fmol/ml)	83.7 ± 11.9	79.7 ± 10.6	86.3 ± 21.1
Tissues (fmol/mg)			
Stomach	413.0 ± 46.7	341.2 ± 66.8	325.0 ± 49.5
Cerebrum	0.05>	0.05>	0.05>
Heart	0.1 ± 0.0	0.05>	0.05>
Kidney	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0

Values are given as the mean ± SEM.
[a] $P < 0.01$ *vs.* control mice.

transgenic mice are shown in Table 3. Eight-week-old male Tg 9–2 and Tg 10–1 mice were significantly shorter in the body length than control mice ($P < 0.05$ and $P < 0.01$, respectively). Female Tg 10–1 mice were significantly shorter than control mice ($P < 0.01$). The difference between female control and Tg 9–2 mice was not significant. BMIs were calculated from the body weights and lengths. No significant difference was noted between control and Tg 9–2 mice and control and Tg 10–1 mice (Table 3). Fifty-two-week-old male Tg 9–2 and Tg10–1 mice were still significantly lighter and shorter, compared with control mice (Table 3), and no significant difference was noted in BMIs between control and transgenic mice. Relative organ weights of 8-wk-old male control and Tg 10–1 mice were calculated from the organ and body weights. No significant difference was noted between control and Tg 10–1 mice (Fig. 2B). No significant difference was noted in the pituitary size between control and Tg 10–1 mice (0.058 ± 0.002 and 0.055 ± 0.003 mg/body weight (grams), respectively).

TABLE 3. Body weights, lengths, and BMIs of 8-wk-old and 52-wk-old control and transgenic mice (n = 8/group)

		Control	Tg 9-2	Tg 10-1
Male				
8-wk-old	Body weight (g)	23.2 ± 0.5	21.0 ± 0.7[a]	16.6 ± 0.6[b]
	Nose-to-anus length (cm)	9.2 ± 0.3	8.6 ± 0.1[a]	7.7 ± 0.3[b]
	BMI (g/cm^2)	27.1 ± 1.2	28.1 ± 3.1[c]	27.3 ± 1.9[c]
52-wk-old	Body weight (g)	34.7 ± 0.8	31.2 ± 0.6[a]	28.4 ± 0.1[b]
	Nose-to-anus length (cm)	10.1 ± 0.1	9.5 ± 0.3[a]	9.1 ± 0.1[b]
	BMI (g/cm^2)	34.4 ± 0.8	34.7 ± 0.6[c]	34.5 ± 0.8[c]
Female				
8-wk-old	Body weight (g)	16.6 ± 1.2	18.7 ± 0.7[c]	10.7 ± 1.1[b]
	Nose-to-anus length (cm)	8.1 ± 0.6	8.5 ± 0.2[c]	6.4 ± 0.2[b]
	BMI (g/cm^2)	25.8 ± 1.8	26.2 ± 1.2[c]	26.5 ± 1.5[c]
52-wk-old	Body weight (g)	25.3 ± 1.3	24.8 ± 0.8[c]	19.6 ± 0.7[b]
	Nose-to-anus length (cm)	9.0 ± 0.4	8.9 ± 0.3[c]	7.9 ± 0.2[b]
	BMI (g/cm^2)	30.9 ± 1.0	31.2 ± 0.7[c]	31.3 ± 1.1[c]

Values are given as the mean ± SEM.
[a] $P < 0.05$ *vs.* control mice.
[b] $P < 0.01$ *vs.* control mice.
[c] Not significant.

Immunohistochemical staining for total and acylated ghrelin of the pituitary

Immunohistochemical staining for total and acylated ghrelin is shown in Fig. 3, A–D. None of total ghrelin-positive cells were observed in the pituitary of control mice (Fig. 3A). On the other hand, many total ghrelin-immunoreactive pituitary cells were observed in Tg 10–1 mice (Fig. 3B). Approximately 30% of the anterior pituitary cells in all sections examined were total ghrelin immunoreactive. None of acylated ghrelin-positive cells were observed in the pituitary of either control or Tg 10–1 mice (Fig. 3, C and D).

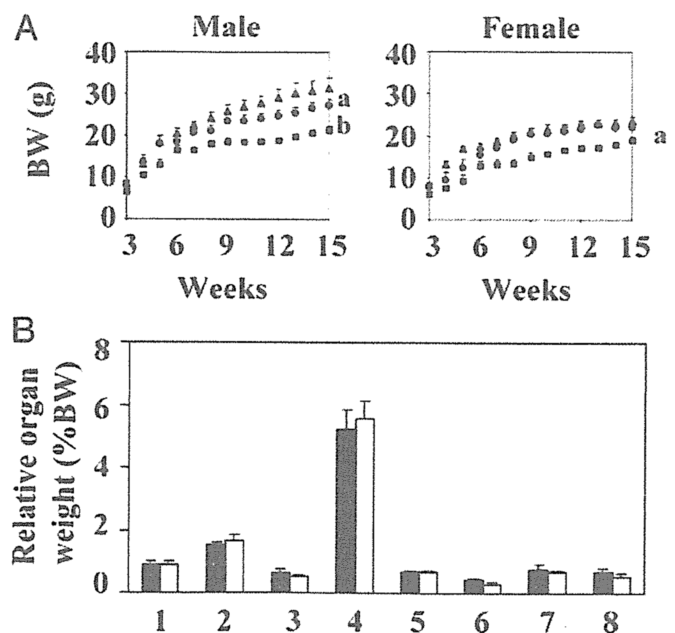

FIG. 2. Body weights (BW) and relative organ weights. A, Body weights of male (*left panel*) and female (*right panel*) control (*triangles*), Tg 9–2 (*circles*), and Tg 10–1 (*squares*) mice (n = 8/group). B, Relative organ weights of 8-wk-old control (*closed bars*) and Tg 10–1 (*open bars*) mice calculated from the organ and body weights (n = 8/group). 1, stomach; 2, cerebrum; 3, heart; 4, liver; 5, kidney; 6, spleen; 7, pancreas; 8, epididymal fat. a, $P < 0.05$; b, $P < 0.01$ (*vs.* control mice).

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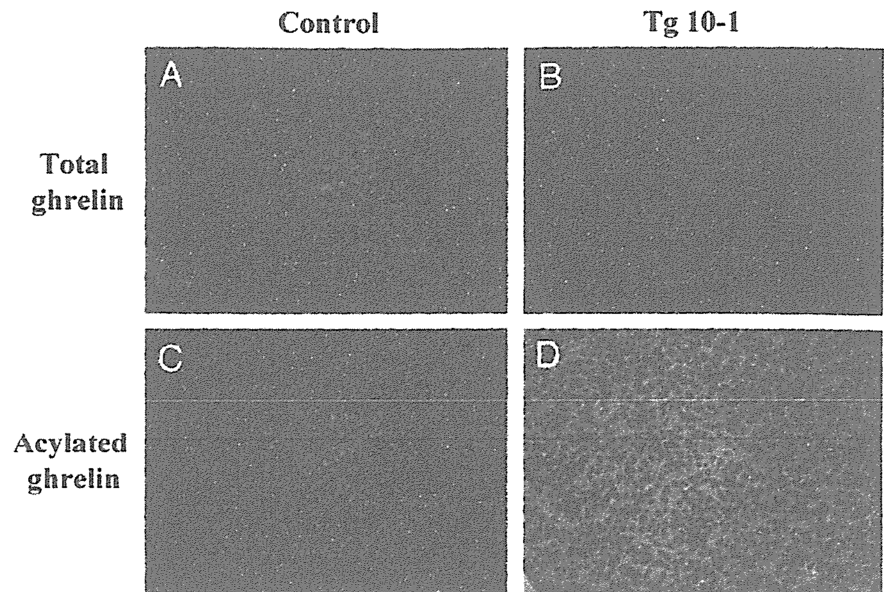

FIG. 3. The localization of total and acylated ghrelin-immunoreactive cells in the pituitary of 8-wk-old male control (A and C) and Tg 10–1 (B and D) mice. A and B, An antiserum raised to ghrelin(13–28) recognizing total (acylated plus des-acyl) ghrelin was used. C and D, An antiserum raised to ghrelin(1–11) specifically recognizing acylated ghrelin was used. Original magnification, ×40. The immunoreactive cells are stained *brown* by the avidin-biotin complex methods.

Food intake and biochemical parameters in the blood

Although absolute amounts of daily food intake were reduced in Tg 9–2 and Tg 10–1 mice, the amounts per body weight were not significantly changed in either male or female Tg 9–2 or Tg 10–1 mice, compared with control mice (Table 4). No significant differences in blood glucose, serum total protein, total cholesterol, and insulin levels were noted between 8-wk-old control and Tg 9–2 mice and control and Tg 10–1 mice (Table 4).

Serum GH, IGF-1, and pituitary GH mRNA levels

Serum GH levels in male control, Tg 9–2, and Tg 10–1 mice were 5.5 ± 1.9, 3.7 ± 0.7, and 2.3 ± 0.9 ng/ml, respectively (Fig. 4A). Those in female control, Tg 9–2, and Tg 10–1 mice were 4.7 ± 1.7, 2.5 ± 0.9, and 1.7 ± 0.8 ng/ml, respectively (Fig. 4A). There were tendencies for decline in serum GH levels in male and female Tg 9–2 and Tg 10–1 mice, compared with control mice, although the differences between them were not significant. Serum IGF-I levels in male control, Tg 9–2, and Tg 10–1 mice were 522 ± 23.6, 413.2 ± 49.0, and 364.1 ± 25.6 ng/ml, respectively (Fig. 4B). Those in male Tg 9–2 and Tg 10–1 mice were significantly reduced, compared with those in control mice ($P < 0.01$ for each). Serum IGF-I levels in female control, Tg 9–2, and Tg 10–1 mice were 509.7 ± 43.1, 545.5 ± 64.1, and 253.7 ± 36.4 ng/ml, respectively (Fig. 4B). Those in female Tg 10–1 mice were significantly reduced, compared with those in control mice ($P < 0.01$). The difference between female control and Tg 9–2 mice was not significant.

Pituitary GH mRNA levels in male control, Tg 9–2, and Tg 10–1 mice were 1.00, 0.62, and 0.42 AU, respectively. Those in Tg 9–2 and Tg 10–1 mice were significantly reduced, compared with those in control mice ($P < 0.05$ and $P < 0.01$, respectively). Pituitary GH mRNA levels in female control, Tg 9–2, and Tg 10–1 mice were 1.00, 0.97, and 0.71 AU. Those in female Tg 10–1 mice were significantly reduced, compared with those in control mice ($P < 0.05$). The difference between those in female control and Tg 9–2 mice was not significant (Fig. 4C).

Plasma ACTH, serum TSH, LH, and FSH levels

Plasma ACTH, serum TSH, LH, and FSH levels in 8-wk-old in male control and transgenic mice are shown in Table

TABLE 4. Daily food intake, blood glucose, serum total protein, total cholesterol, and insulin levels in 8-wk-old control and transgenic mice (n = 8/group)

	Control	Tg 9-2	Tg 10-1
Male			
Daily food intake (mg/BW/d)	149.1 ± 7.6	154.2 ± 3.2	155.2 ± 5.9
Serum total protein (mg/dl)	5.1 ± 0.2	4.9 ± 0.1	5.5 ± 0.1
Serum total cholesterol (mg/dl)	121.0 ± 12	116.9 ± 11.3	123.1 ± 8.3
Blood sugar (mg/dl)	134.2 ± 9.4	135.3 ± 8.7	136.7 ± 5.8
Serum insulin (pg/ml)	3233 ± 407	4624 ± 1015	2419 ± 423
Female			
Daily food intake (mg/BW/d)	167.5 ± 2.3	169.5 ± 4.7	165.8 ± 9.8
Serum total protein (mg/dl)	5.4 ± 0.1	5.1 ± 0.3	5.2 ± 0.3
Serum total cholesterol (mg/dl)	129.0 ± 9.3	123.4 ± 9.2	122.9 ± 6.1
Blood sugar (mg/dl)	132.1 ± 5.5	134.2 ± 6.4	130.6 ± 7.2
Serum insulin (pg/ml)	1182 ± 284	2079 ± 587	1799 ± 725

Values are given as the mean ± SEM. BW, Body weight.

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