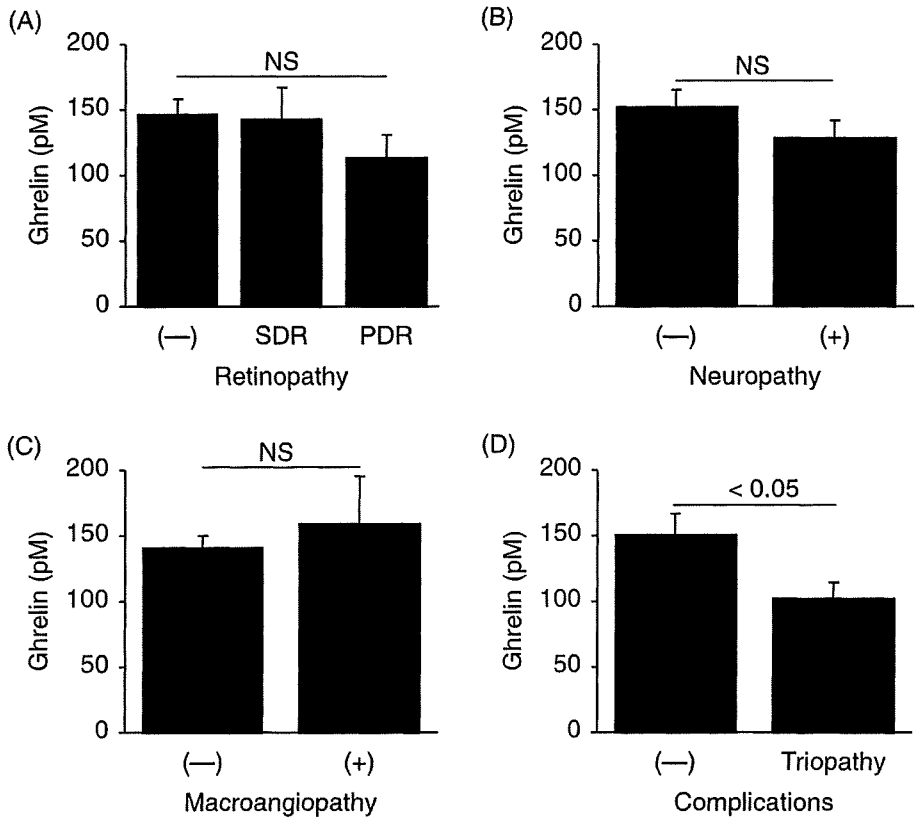

Fig. 3. Plasma ghrelin concentrations in normal-weight diabetic patients with or without diabetic complications except for those with stage 4 nephropathy. Plasma ghrelin concentrations were related to the presence or absence of retinopathy (A), neuropathy (B), macroangiopathy (C), or diabetic triopathy (D). Data represent the means ± SEM. NS: not significant.

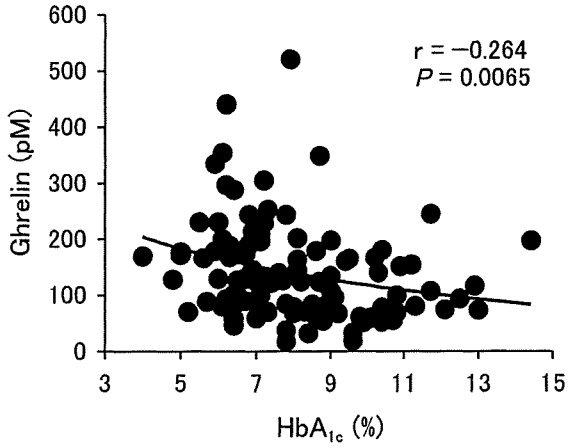

Fig. 4. Correlation between plasma ghrelin concentration and HbA_{1c} level in diabetic patients.

Table 2. Stepwise multiple regression analysis with plasma ghrelin level as the dependent variable, and age, sex, BMI, HbA_{1c}, fasting plasma glucose, and serum creatinine as independent variables

Independent variables	Dependent variable: Ghrelin		
	r	β	*P* value
Step 1; Serum creatinine	0.326	0.326	0.0009
Step 2; BMI	0.420	−0.265	<0.0001
Step 3; HbA_{1c}	0.467	−0.213	<0.0001

BMI, body mass index

There were no significant differences in suppression rates of plasma ghrelin at 30 and 60 min after a test meal between patients with normal $CV_{R\text{-}R}$ values and patients with low $CV_{R\text{-}R}$ values (Fig. 5B). Postprandial plasma ghrelin concentrations were not different depending on the treatments (*i.e.*, insulin therapy, oral hypoglycemic agents, and diet alone) (data not shown).

Discussion

This study shows that the plasma ghrelin concentrations in diabetic patients with long-term poor glycemic

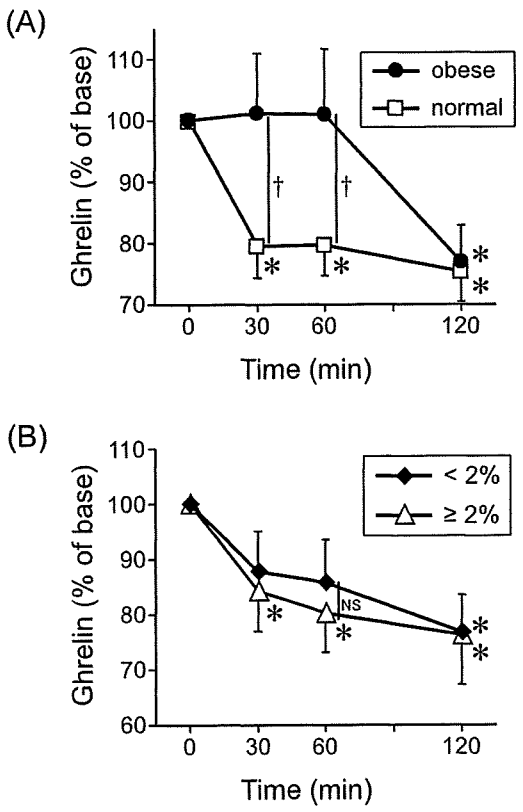

Fig. 5. Suppression of plasma ghrelin concentration after a test meal. (A) Changes in plasma ghrelin concentrations in normal-weight (□) and obese (●) patients. (B) Changes in plasma ghrelin concentrations in patients with normal $CV_{R\text{-}R}$ (≥2%) (△) or low $CV_{R\text{-}R}$ (<2%) (◆) values. *P<0.05 vs. basal value in same group. †P<0.05 vs. obese group. NS: not significant.

control were lower than those in patients with good glycemic control. In addition, the postprandial decrease in the level of ghrelin was significantly smaller in obese patients.

Yoshimoto *et al.* reported that the plasma ghrelin concentration in patients with mild to severe chronic renal failure was positively correlated with s-Cre, and negatively correlated with the Ccr [21]. Moreover, the plasma ghrelin concentrations in patients with end-stage renal diseases were reduced by 50% after a single course of hemodialysis [21]. Bilateral nephrectomy markedly increased the plasma ghrelin concentration without a significant increase in the ghrelin mRNA level in the stomach of mice [21]. Therefore, the elevated plasma ghrelin concentration in patients with stage 4 nephropathy is considered to be associated with decreased clearance of ghrelin in the kidney.

No correlation between the levels of ghrelin and HbA_{1c} was reported in type 1 diabetic children [22, 23]. We found low plasma ghrelin concentrations in diabetic patients with high HbA_{1c} levels or with diabetic triopathy, a condition that results in long-term poor glycemic control. This finding suggests that the plasma ghrelin concentration may be suppressed by long-term poor glycemic control. The effect of ghrelin on insulin secretion is controversial. Some studies have reported a stimulatory effect, whereas others reported an inhibitory effect [24–27]. Conversely, the plasma ghrelin concentration was suppressed in a hyperinsulinemic euglycemic clamp study in normal subjects, and intensive insulin therapy in type 1 diabetic patients [28, 29]. In addition, ghrelin administration induced an increase in glucose levels in human [26, 30]. The plasma ghrelin concentration was negatively correlated with the serum insulin level and insulin resistance [11]. The relationships between the levels of ghrelin and the levels of insulin, glucose, or insulin resistance are complex. This study found no significant correlations between the plasma ghrelin concentration and the fasting glucose level, the fasting insulin level except for that in patients receiving insulin therapy, or the fasting C-peptide level, as well as the homeostasis model assessment score (data not shown). Due to the subjects' various insulin secretion capacities, insulin resistance levels, and different treatments they received, the mechanisms underlying the negative correlation between the HbA_{1c} level and the plasma ghrelin concentration cannot be explained.

In this study, there were no significant differences in the plasma ghrelin concentrations in diabetic patients with and without macroangiopathy and retinopathy. There is data suggesting that ghrelin may have a favorable effect on endothelial function [16–18]. Low plasma ghrelin concentrations in diabetic patients with poor glycemic control may be a risk factor for the progression of micro- and macro-vascular complications.

$CV_{R\text{-}R}$ is a simple index of diabetic autonomic neuropathy, in particular of vagal neuropathy, and a $CV_{R\text{-}R}$ value of 2% has been recognized as a critical level for vagal neuropathy [31, 32]. The vagal nerve conveys ghrelin-mediated signals for GH secretion and food intake from the stomach to the brain [33]. Circulating ghrelin levels in humans were increased and reduced by cholinergic agonists and antagonists, respectively [34]. A postprandial decrease in the ghrelin level was not found in sheep treated with cholinergic blockers [35]. While there was no significant difference in the

postprandial suppression rates of ghrelin between the patients with normal CV_{R-R} values and those with low CV_{R-R} values, a significant postprandial decrease in ghrelin levels was detected only in patients with normal CV_{R-R} values at 30 and 60 min after a test meal. This finding supports the importance of the vagal nerve in the regulation of ghrelin secretion, which will be proved more clearly if some new, simple, and accurate methods to examine diabetic autonomic neuropathy are developed.

In conclusion, the present study demonstrates that ghrelin secretion may be suppressed by long-term poor glycemic control and that obesity may influence the regulation of ghrelin secretion.

Acknowledgments

We would like to thank Dr. H. Nakao for his helpful discussions. This study was supported in part by the 21st Century COE Program and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Ministry of Health, Labor, and Welfare of Japan; the Takeda Medical Research Foundation; the Foundation for Growth Science; and the Fujisawa Foundation to M.N.

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Acute Incremental Exercise Decreases Plasma Ghrelin Level in Healthy Men

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received 04.12.2006
accepted 27.03.2007

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DOI 10.1055/s-2007-991177
Horm Metab Res 2007;
39: 849–851

ISSN 0018-5043

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Introduction

Ghrelin, a 28 amino acid peptide, was originally isolated as an endogenous ligand for growth hormone (GH) secretagogue receptor (GHS-R) from human and rat stomach [1]. This peptide strongly stimulates GH secretion and food intake [1]. Plasma ghrelin levels increase following fasting and decrease after oral and intravenous glucose administration, suggesting that ghrelin is upregulated under conditions of negative energy balance and downregulated in the setting of positive energy balance [2].

The systemic circulation during acute exercise is regulated by the attenuation of vagal efferent activity, known as Bainbridge reflex, and an increase of sympathetic nerve activity. A vagal efferent pathway mediates ghrelin release [3,4]. In addition, gastric vagal afferent serves as the major pathway conveying ghrelin's signals for starvation and growth hormone secretion to the brain [5]. Moreover, ghrelin exhibits GHS-R-independent biological activities, including a cytoprotective effect on cultured cardiomyocytes and a proliferative effect on human osteoblast [6,7]. Thus, ghrelin plays a role in cell survival through anabolic processes. As exercise stress induces a variety of catabolic processes, involving glycolysis, lipolysis, and protein degradation [8]. We assume that ghrelin release and ghrelin action may change in response to the alternation of the autonomic activity and/or catabolic state during an acute exercise.

A number of studies have reported the relationship between plasma ghrelin levels and exercise [9–14]. Although acute aerobic exercise such as running and cycling exercise is not likely to alter plasma ghrelin levels [9,11,12,14], acute resistance exercise induces a decline in circulating ghrelin levels [10,13]. These findings suggest that mechanical stress to muscles or the metabolic state may influence the plasma ghrelin concentrations. However, the relationship between physical exercise and changes in plasma ghrelin levels has yet to be defined. We investigated the effect of energy consumption including metabolic changes on plasma ghrelin level using incremental exercise protocol, in which each stage was set for 10 minutes for stabilizing the relationship between exercise intensity and hormonal parameters.

Material and Methods

We examined five healthy men (mean age, 26.2 ±0.5 years; mean height, 172.6±0.7 cm; mean weight, 69.4±0.7 kg; average BMI, 23.3±0.2 kg/m^2, and maximum oxygen consumption ($\dot{V}O_2$max), 43.8±0.8 ml/min/kg), who had not engaged in any regular exercise for at least two years. All individuals were healthy, nonsmokers, and nonobese and were not currently taking any medications. Prior to beginning of the study, the nature, purpose, and risks of the study were explained to all subjects and their written informed consent was obtained. The study, approved by the local Ethics Committee of the Fukuoka University, was conducted in accordance with the Helsinki Declaration.

At baseline, all subjects underwent an exercise test on a cycle ergometer (STB-1350, Nihon Kohden, Tokyo, Japan) to determine exercise intensity. The work rate, initially set at 15 W, was increased every minute by 15 W. The test was continued until subjective exhaustion was achieved. $\dot{V}O_2$max was calculated with a respirometer (Fukuda Irika CR-20, Tokyo, Japan). Blood samples were obtained from the earlobe every 30 seconds to measure blood lactate levels.

*These authors contributed equally to this work.

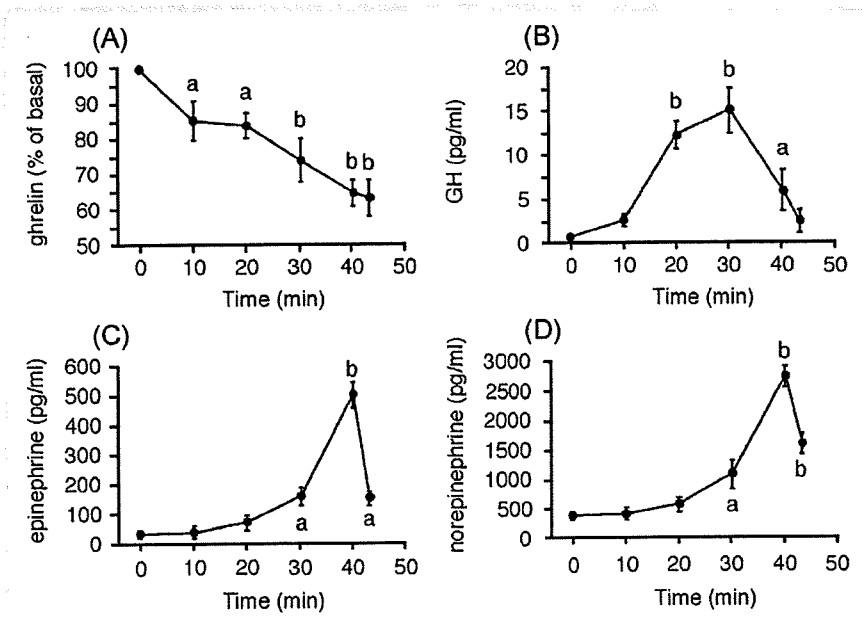

Fig. 1 Plasma levels of ghrelin (**A**), GH (**B**), epinephrine (**C**), and norepinephrine (**D**) during and after incremental exercise. Data are the means ± SE. a: $p<0.05$; b: $p<0.001$ in comparison with basal levels.

The lactate threshold (LT) and onset of blood lactate accumulation (OBLA) were determined for each subject.

After an overnight fast, subjects exercised progressively every 10 minutes at four different intensities: half of the LT (1/2LT), the LT, the OBLA, and the OBLA-peak (a midpoint of OBLA and $\dot{V}O_2max$). Blood sampling was performed from the antecubital vein prior to exercise, during the four different exercise intensities (just before the end of each stage), and 3 minutes into the recovery period. The $\dot{V}O_2max$ measurement and the incremental exercise testing periods were separated by 5 days to ensure complete recovery.

Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA.2Na (1 mg/ml) and aprotinin (500 U/ml) and centrifuged at 4 °C. A 10% volume of hydrochloric acid (1 M) was added to the isolated plasma samples immediately after separation of plasma. Samples were stored at −30 °C until assayed. Plasma ghrelin was measured by radioimmunoassay [2]. Serum GH concentrations were analyzed using a commercially available GH ELISA kit (Biocode SA, Liege, Belgium). Plasma epinephrine and norepinephrine were assayed by high-performance liquid chromatography (SRL Inc, Tokyo, Japan).

Statistical differences were analyzed by the ANOVA factorial with Fisher's PLSD. Simple linear regression analysis was used to estimate the relationship between the percent change in ghrelin and other measured parameters. All values are shown as means ± SE. A p value of <0.05 was considered to be statistically significant.

Results

Basal fasting plasma ghrelin concentrations averaged 139.9 ± 12.4 fmol/ml. Plasma ghrelin levels decreased significantly during exercise at the 1/2 LT ($p<0.05$), the LT ($p<0.05$), the OBLA ($p<0.001$), and the OBLA-peak ($p<0.001$) (○ **Fig. 1A**). Plasma ghrelin levels remained below pre-exercise level 3 minutes into the recovery period ($p<0.001$; ○ **Fig. 1A**). Plasma GH concentrations increased significantly at the LT exercise intensity ($p<0.001$), peaking at the OBLA ($p<0.001$; ○ **Fig. 1B**). Plasma epinephrine concentrations increased significantly during exercise at the OBLA ($p<0.05$) and the OBLA-peak ($p<0.001$) intensities and decreased 3 minutes into the recovery period, although these values remained well above pre-exercise level ($p<0.05$; ○ **Fig. 1C**). Plasma norepinephrine also increased during exercise at the OBLA ($p<0.05$) and OBLA-peak ($p<0.001$) intensities. Although norepinephrine levels decreased in the recovery period, these values remained well above pre-exercise level ($p<0.001$; ○ **Fig. 1D**). We observed significant negative correlations between the change in plasma ghrelin and both plasma epinephrine ($r=-0.533$, $p<0.05$) and norepinephrine ($r=-0.607$, $p<0.001$) levels measured during exercise. Plasma ghrelin level tended to be in negative correlation with GH level during exercise ($r=-0.379$, $p=0.0616$).

Discussion

This is the first study to report the significant decrease of plasma ghrelin concentrations during acute endurance exercise. Ghrelin is an endogenous ligand for the GH secretagogue receptor and it strongly stimulates GH release through a mechanism independent from hypothalamic GHRH [1]. We have examined, thus, the effect of acute exercise on GH and plasma ghrelin. We have demonstrated that plasma GH levels robustly increased during exercise, whereas plasma ghrelin levels decreased. No correlation existed between plasma ghrelin and GH during exercise. These observations correlate well with previous reports that plasma ghrelin is not involved in exercise-induced GH secretion [9–13]. Some studies have examined the relationships between running and cycling exercise and plasma ghrelin concentrations. Schmidt et al. [11] could not identify an association between plasma ghrelin and either low, moderate, or intense treadmill running. Dall et al. [9] also reported that cycling exercise had no effect on plasma ghrelin levels. Pomerants et al. [14] reported that moderate acute aerobic exercise did not change serum ghrelin level in boys at different pubertal stages. However, Kraemer et al. [10] indicated a decrease in plasma ghrelin following resistance exer-

cise with concentric muscle contraction. More recent study demonstrated that a circuit resistance exercise at 60% of one repetition maximum led to a suppression of plasma ghrelin concentrations [13]. The reasons underlying the discrepancy between these studies, including our results, are unclear. Cycle ergometer exercise needs the concentric muscle contractions in legs because of the resistance of the pedals. Therefore, a decrease in plasma ghrelin during incremental exercise may be explained by an increase in muscle contractions in subjects of our study. Dall et al. [9] also speculated that GH may inhibit systemic ghrelin release resulting from lower ghrelin levels during GH replacement in GH-deficient patients. Plasma ghrelin levels tended to be in a negative correlation with GH levels during exercise in this study, suggesting that exercise-induced GH release may inhibit ghrelin release by a negative feedback system.

We observed negative correlations between plasma ghrelin and both plasma epinephrine and norepinephrine levels. Previous studies have suggested that the adrenergic system is involved in the maintenance of gastric mucosal integrity [15]. Ghrelin is produced primarily in the distinct type of endocrine cells in the gastric oxyntic glands [16]. It has been shown that fasting plasma ghrelin levels are regulated by catecholamines [17]. After exercise, reduction in portal venous flow is also related to the degree of sympathoadrenergic activation [18]. Treadmill exercise for 30 minutes in rats reduced the stomach blood flow by 85% [19]. Exercise induces the development of reduction of blood flow in the gastric mucosa, which is dependent on exercise intensity [20]. Changes in plasma ghrelin concentration during exercise may be induced by sympathetic activation resulting in a decline of stomach blood flow [18–20]. Based on the evidences that the secretion and action of ghrelin depend on the vagal pathway and the distribution of blood stream is altered by the autonomic action during exercise, we suggest that the adrenergic system may play a role, at least indirectly, in the regulation of ghrelin release into the systemic circulation during exercise.

Circulating ghrelin functions as anticatabolic agent in peripheral tissues, involving adipogenesis, osteogenesis, and cell proliferation via the interactions with the target proteins on membranes of cadiomyocytes, adipocytes, and osteocytes [6,(7]. Skeletal muscle requires plenty of fuel to maintain muscle contraction during exercise. A part of the fuel during exercise is supplied by degradation of intramuscular glycogen and triglyceride, suggesting that skeletal muscle during exercise is potently dominated by catabolic process [8]. Therefore, decreased plasma ghrelin may represent an increase in the ghrelin utilization under catabolic-anabolic imbalance in peripheral tissues during exercise.

Our results indicate that acute exercise reduced plasma ghrelin levels. In addition, plasma ghrelin correlated inversely with plasma epinephrine and norepinephrine. The ghrelin system and distribution of blood stream are altered by the autonomic state. Therefore, the decrease in plasma ghrelin levels during exercise may be partially explained by the autonomic changes. In addition to an increase in feeding and GH secretion, ghrelin functions as an anabolic agent in peripheral tissues. Although the reasons underlying the discrepancy between previous studies using aerobic exercise and the present study are unclear, our results may suggest that acute aerobic exercise in our protocol suppresses ghrelin secretion and/or enhances ghrelin utilization in peripheral tissues. Further studies, by analyzing GHS-R expression in the skeletal muscle or measure the ghrelin levels in blood samples of the portal vein during exercise, may help clarify a role of ghrelin during exercise.

Acknowledgments

This study was supported in part by The 21st Century COE Program and grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Ministry of Health, Labor and Welfare, Japan; Japan Foundation for Aging and Health; The Foundation for Growth Science in Japan; Novo Nordisk Foundation; Novartis Foundation for Gerontological Research; Society of Molecular Mechanism of Digestive Tract; and Takeda Medical Research Foundation.

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Emerging results of anticatabolic therapy with ghrelin

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Purpose of review

This review summarizes recent developments in research into anticatabolic therapies with ghrelin. Potential conditions in which ghrelin treatment may be useful include cachexia, anorexia and ageing. We highlight a number of intriguing basic topics related to the anticatabolic effects of ghrelin.

Recent finding

Repeated administration of ghrelin to patients with congestive heart failure or chronic obstructive pulmonary disease improved appetite, body composition, muscle wasting and functional capacity in open-label pilot studies. An acute, randomized, placebo-controlled, crossover clinical trial of cancer patients with anorexia revealed marked increases in energy intake following treatment. The effects of ghrelin treatment in patients with anorexia nervosa are controversial. Basic research studies have extended our understanding of the upstream regulation of neuropeptide Y/agouti-related protein signalling and the central control of adipocyte metabolism. In addition, alterations in fat-free mass may play a role in ghrelin regulation.

Summary

A number of studies are currently evaluating the anticatabolic effects of ghrelin in the treatment of various diseases, including cachexia, anorexia and age-related disorders. These studies will hopefully lead to the development of novel clinical applications for ghrelin treatment. These studies have also facilitated a better understanding of the molecular basis of the anticatabolic effects of ghrelin.

Keywords

ageing, anabolism, anorexia, cachexia, catabolism, growth hormone-secretagogue

Curr Opin Clin Nutr Metab Care 10:278–283. © 2007 Lippincott Williams & Wilkins.

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Current Opinion in Clinical Nutrition and Metabolic Care 2007, 10:278–283

Abbreviations

AMPK	adenosine 5′-monophosphate activated protein kinase
CHF	congestive heart failure
COPD	chronic obstructive pulmonary disease
FFM	fat-free mass
GHS-R	growth hormone-secretagogue receptor

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Introduction

Ghrelin is a natural ligand for the growth hormone-secretagogue receptor (GHS-R). This peptide possesses a unique fatty acid modification, an *n*-octanoylation, of Ser 3 [1]. There are two circulating forms of ghrelin: acylated and unacylated (desacyl). Of these, the acylated form is essential for ghrelin's biological activity through the GHS-R. Ghrelin plays a critical role in a variety of physiological processes. It potently stimulates growth hormone secretion, functioning in energy homeostasis by stimulating food intake and promoting adiposity via a growth hormone-independent mechanism [2–4]. Growth hormone is an anabolic hormone, sparing protein stores at the expense of fat during conditions of caloric restriction. Ghrelin also inhibits production of anorectic proinflammatory cytokines [5,6]. Thus, ghrelin exhibits anticatabolic actions through both growth hormone-dependent and independent mechanisms.

Peripheral administration of ghrelin ameliorated body weight loss and anorexia in a rat model of cachexia in the presence of chronic heart failure [7,8], in rodents administered interleukin-1β [9,10], and in a rat model of cancer cachexia [8]. Trials evaluating treatment of various catabolic states with ghrelin are accumulating. This review summarizes recent advances in this area of research.

Basic research

Ghrelin increases appetite by stimulating production of orexigenic neuropeptide Y/agouti-related protein by cells in the arcuate nucleus. Pharmacological administration of ghrelin induces a positive energy balance, increasing body weight [2–4]. Recent studies have furthered our understanding of the upstream regulation of neuropeptide Y/agouti-related protein signalling and the central control of adipocyte metabolism.

Ghrelin and adenosine 5′-monophosphate activated protein kinase

Adenosine 5′-monophosphate activated protein kinase (AMPK) regulates cellular and systemic energy

homeostasis [11••], and it mediates some of the effects of adipocyte hormones including ghrelin, leptin and adiponectin. There is increasing evidence that AMPK plays a central role in the appetite-modulating and metabolic effects of multiple hormones, including ghrelin, leptin, adiponectin and endocannabinoids. AMPK phosphorylation increases neuropeptide Y gene expression [12]. Activation of the hypothalamus increases food intake and body weight, whereas its inhibition has the opposite effects [13]. Ghrelin stimulates hypothalamic AMPK activity following either intraperitoneal [14,15] or intracerebroventricular injection [15], suggesting that AMPK activation may mediate at least part of ghrelin's orexigenic effect [15].

In peripheral tissues, ghrelin modulates AMPK activity differently in several tissues. Ghrelin treatment inhibits AMPK activity in liver and adipose tissue, suggesting that the effect of ghrelin on gluconeogenesis and fat deposition may occur via inhibition of AMPK activity in those tissues [15,16] (Table 1). In the heart, AMPK is activated by ischaemia, functioning to limit the damage and induction of apoptosis associated with ischaemia and reperfusion injury [17]. Kola *et al.* [15] identified a significant increase in AMPK activity in the myocardium following ghrelin administration. This result suggests that AMPK activation may mediate at least some of beneficial effects of ghrelin on cardiovascular function. In contrast, those investigators did not observe any effect of ghrelin on AMPK activity in skeletal muscle [15,16].

Central effect of ghrelin on adipocyte metabolism

The neuroendocrine circuits in the brain that regulate appetite and food intake also function in the control of peripheral metabolism and thermogenesis [18]. Theander-Carrillo *et al.* [19•] demonstrated that long-term central ghrelin infusions increased glucose utilization rate in both white and brown adipose tissue without affecting skeletal muscle mass. In white adipocytes, mRNA expression of various fat storage-promoting enzymes increased markedly, whereas expression of the rate-limiting enzyme in fat oxidation, namely carnitine palmitoyl transferase-1α, was decreased. In brown adipocytes, central ghrelin infusion reduced the expression of thermogenesis-related mitochondrial uncoupling proteins 1 and 3. These effects of ghrelin, which occurred independently of ghrelin-induced hyperphagia, appear to be mediated by the sympathetic nervous system. Thus, centrally produced ghrelin functions physiologically in the control of adipose tissue cell metabolism.

Table 1 Effect of ghrelin on AMPK activity in different tissues (adapted from Kola *et al.* [11••])

	AMPK activity	Effect	Reference
Hypothalamus	Stimulate	↑ Appetite	[14,15]
Heart	Stimulate	↑ Glucose uptake	[15,17]
Liver	Inhibit	↑ Glucogenesis	[15,16]
Adipose tissue	Inhibit	↑ Fat accumulation	[15,16]
Skeletal muscle	No change	–	[15,16]

AMPK, adenosine 5′-monophosphate activated protein kinase.

Clinical research

Trials attempting to exploit the anticatabolic effects of ghrelin in treatment for various disease states have been accumulating. These studies have sought to evaluate ghrelin as a treatment for patients with cachexia, anorexia and age-related disorders. Cachexia manifests as excessive weight loss in the setting of an underlying chronic disease such as congestive heart failure (CHF), cancer, chronic obstructive pulmonary disease (COPD), or severe inflammation [20]. Anorexia is one of the major causes of weight loss in cachexia. Loss of appetite and weight loss are the major causes of morbidity and mortality in patients with anorexia–cachexia syndrome. There is an immediate need for more effective and better tolerated treatments to stimulate appetite [21]. Several trials have already attempted to explore the application of ghrelin treatment in patients with cachexia. Circulating ghrelin levels are elevated in patients with cachexia in comparison with those in normal-weight control individuals, reflecting the negative energy balance state [22–25].

Cachexia in congestive heart failure

Ghrelin has protective cardiovascular effects and induces a positive energy balance state through both growth hormone-dependent and independent mechanisms [26•]. Growth hormone treatment may be useful in the subgroup of patients with cardiac cachexia [27]. Ghrelin, however, stimulates food intake, induces adiposity, acts on the central nervous system to decrease sympathetic nerve activity, and inhibits apoptosis of cardiomyocytes and endothelial cells in a growth hormone-independent manner. Nagaya *et al.* [28] investigated the effects of ghrelin on cardiac cachexia in patients with CHF (Table 2). Three weeks of administration of ghrelin increased both food intake and body weight. Moreover, this study demonstrated improvements in exercise capacity, muscle wasting and left ventricular function. Ghrelin treatment also resulted in significant decreases in plasma noradrenaline (norepinephrine). Although the study was neither randomized nor placebo-controlled, eight CHF patients who did not receive ghrelin (control group) were also followed to rule out any time-course effects during hospitalization. All of the aforementioned parameters remained unchanged in the patients with CHF who did not receive ghrelin therapy. Further studies will be necessary to identify the exact pathways involved and to determine the best therapeutic strategies to combat the wasting process in cardiac cachexia with ghrelin [27].

Cachexia in chronic obstructive pulmonary disease

Patients with COPD often exhibit some degree of cachexia [29]. Cachexia is an independent risk factor

Table 2 Summary of prior clinical studies examining anticatabolic therapy with ghrelin

Diseases	Reference	Year	Study design	Ghrelin injection
CHF	[28]	2004	Open-label pilot study	2 μg/kg twice daily intravenous, for 3 weeks
COPD	[31]	2005	Open-label pilot study	2 μg/kg twice daily intravenous, for 3 weeks
Cancer cachexia	[32]	2004	Acute, randomized, placebo-controlled, crossover study	17 ng/kg per min intravenous, for 270 min
Anorexia nervosa	[38•]	2006	Acute interventional study	5 pmol/kg per min intravenous, for 300 min
Anorexia nervosa	[39]	2004	Acute interventional study	1.0 μg/kg intravenous
ESRD	[54]	2005	Acute, randomized, placebo-controlled, crossover study	3.6 nmol/kg subcutaneous

CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; ESRD, end-stage renal disease.

for mortality in COPD; growth hormone treatment has been demonstrated to increase muscle mass in such patients. COPD and CHF share multiple pathophysiological disturbances, including anaemia and neurohormonal activation [30•]. The ability of ghrelin to improve cachexia and functional capacity in patients with COPD was examined in an open-label pilot study [31]; ghrelin was administered intravenously for 3 weeks to seven cachectic patients with COPD. Repeated administration of ghrelin significantly increased food intake, body weight, lean body mass, and peripheral and respiratory muscle strength. Ghrelin ameliorated the exaggerated sympathetic nerve activity, as indicated by marked decreases in plasma noradrenaline (norepinephrine) levels. Treatment with ghrelin improved appetite, body composition, muscle wasting, functional capacity, and sympathetic augmentation in cachectic patients with COPD. Future studies should attempt to define which patients are most likely to benefit from ghrelin treatment. Comparisons of this treatment with current standard medications are required [30•].

Cachexia in cancer

Anorexia, frequently encountered in cancer patients, is a major cause of malnutrition and cachexia in this patient population. Nearry *et al.* [32] performed an acute, randomized, placebo-controlled, crossover clinical trial to determine whether ghrelin could stimulate appetite in seven cancer patients with anorexia. A marked increase in energy intake was observed after ghrelin infusion in comparison with saline control individuals; all patients in the study exhibited increased food consumption. The meal appreciation score was also higher in ghrelin-treated individuals.

Tumour-bearing mice (MCG101) exhibit anorexia, fat loss and muscle wasting due to increased levels of prostaglandin E_2 and proinflammatory cytokines (interleukin-1β, interleukin-6 and tumour necrosis factor-α). Ghrelin treatment of these animals increased food intake, body weight and whole body fat at both low and high doses in normal control animals, whereas tumour-bearing mice exhibited improvements in intake and body composition only at the high dose of ghrelin [33]. Exogenous ghrelin normalized GHS-R expression in hypothalamus of tumour-bearing mice without altering ghrelin expression in the gastric fundus. Tumour growth was not altered by exogenous ghrelin treatment. These results suggest that MCG 101 mice became ghrelin-resistant despite upregulation of hypothalamic GHS-R expression.

Ghrelin resistance in cachexia was suggested by measurement of plasma ghrelin levels in cancer patients. Garcia *et al.* [23] observed that active ghrelin levels and the ratio of active to total ghrelin were significantly increased in patients with cancer-induced cachexia in comparison with control patients with cancer and healthy control individuals. Appetite, as measured using a visual–analogue scale, was not increased in patients with cachexia. These results suggest that cachexia may be a state of ghrelin resistance, because appetite does not correlate with ghrelin levels. The observed changes in the ratio of active to total ghrelin suggest that a mechanism other than increased secretion is responsible for the increase in active ghrelin. In fact, elevation in circulating ghrelin levels has been reported by others [25,34].

Anorexia nervosa and related disorders

Anorexia nervosa is an eating disorder characterized by self-induced starvation, leading to chronically decreased caloric intake. Plasma ghrelin levels are elevated in lean patients with anorexia nervosa, which is consistent with a negative energy balance state [35–37]. Few preliminary studies have examined the effects of ghrelin on individuals with anorexia nervosa. Miljic *et al.* [38•] infused ghrelin into nine patients with anorexia nervosa with very low body weights, six patients with anorexia nervosa who had partially recovered their original body weight but were still amenorrhoeic, and 10 constitutionally thin women. After infusion, the 15 patients with anorexia nervosa felt significantly less hungry in comparison with the constitutionally thin women, suggesting that patients with anorexia nervosa are resistant to the orexigenic effects of ghrelin in comparison with healthy control individuals. In an independent study, however, six out of nine patients with restrictive anorexia nervosa exhibited increased hunger after ghrelin administration, which was a similar proportion to that seen in normal individuals (five out of seven) [39]. Clearly, further studies, including randomized controlled trials, are needed to determine the utility of ghrelin treatment for anorexia nervosa.

Functional dyspepsia is a disorder characterized by chronic or recurrent upper abdominal pain or discomfort [40]. Although no specific organic abnormalities are thought to be present in functional dyspepsia, abnormalities in gastrointestinal motility and sensitivity may play a role in its pathogenesis in a large subgroup of patients. A number of patients also suffer from anorexia and weight loss. Therefore, we are currently examining whether repeated ghrelin administration can increase food intake in patients with functional dyspepsia. We found that plasma levels of acylated, but not desacyl, ghrelin correlated with subjective symptom score in patients with functional dyspepsia, suggesting that acylated ghrelin may function in the pathophysiology of this disorder [41].

Ageing

Elderly individuals would also be suitable candidates for ghrelin treatment. Ageing is associated with progressive decreases in growth hormone secretion, appetite and energy intake [42–45]. This reduction in growth hormone secretion, termed 'somatopause', may be a cause of the age-related metabolic and physiological changes that lead to reduced lean body mass and expansion of adipose mass. In the elderly, sarcopenia is associated with functional decline and death. This population also exhibits altered blood lipid profiles that favour the development of vascular disease, leading to increased overall mortality. This age-related reduction in energy intake, which has been termed 'the anorexia of ageing', predisposes to the development of malnutrition. Such nutritional deficiency has been implicated in the development and progression of chronic diseases that commonly affect the elderly and in the observed increases in mortality in this population. Growth hormone therapy increases insulin-like growth factor-I levels, promotes anabolism and increases muscle strength in healthy elderly individuals and in selected patient groups [46–48]. Therefore, both ghrelin and GHS may have therapeutic potential to assist in the recovery of frail patients who require both nutritional support and conventional rehabilitation [49].

We found that the plasma levels of acylated ghrelin in healthy elderly women tended to be low. These values correlated positively with insulin-like growth factor-I levels, suggesting that the negative feedback regulation of ghrelin expression does not function properly in elderly individuals [50]. Because acylated ghrelin concentrations in elderly women correlated with both systolic blood pressure and frequency of bowel movements, acylated ghrelin may play a role in regulation of the growth hormone/insulin-like growth factor-I axis and blood pressure, and control of bowel movements.

End-stage renal disease

End-stage kidney disease is a chronic condition that is frequently associated with nutritional dysfunction [51•]. This type of malnutrition, which is resistant to intervention, is a major predictor of morbidity and mortality on either peritoneal dialysis or haemodialysis. Uraemic patients with anorexia exhibited lower ghrelin plasma levels than those in obese patients or patients with a normal appetite [52]. Plasma levels of active ghrelin in patients with end-stage renal disease were decreased after a single course of haemodialysis [53]. Wynne *et al.* [54] aimed to determine whether ghrelin administration could enhance food intake in patients with evidence of malnutrition who receive maintenance peritoneal dialysis. Nine peritoneal dialysis patients with mild to moderate malnutrition were administered (subcutaneously) either ghrelin or a saline placebo according to a randomized, double-blind, crossover protocol. Ghrelin administration significantly increased the mean absolute energy intake of the group during the study meal and maintained the nonsignificant increases observed in energy intake over the first 24 h after intervention without a subsequent rebound. Thus, ghrelin administration may also be able to enhance short-term food intake in dialysis patients with mild to moderate malnutrition.

Plasma ghrelin levels and fat-free mass

Recent reports have suggested that alterations in fat-free mass (FFM), in conjunction with diet-induced weight loss, may alter ghrelin regulation. In obese individuals, increased 24-h ghrelin levels after weight loss correlated with decreases in body mass index, subcutaneous fat and FFM, whereas they did not correlate with changes in fat mass, fat cell size, leptin, insulin, insulin sensitivity, lipid levels, or free fatty acids [55•]. The change in FFM correlated with elevations in ghrelin levels independent of body adiposity. In support of this finding, healthy elderly individuals also exhibit basal and postprandial ghrelin increases with changes in FFM, specifically skeletal muscle mass reduction [56•]. Changes in ghrelin levels may thus serve as an integrative signal, reflecting the changes in FFM to hypothalamic centres controlling energy homeostasis.

Conclusion

Consistent with the wide expression patterns of both ghrelin and its receptor, this peptide functions in a number of different physiological processes, including anticatabolic effects. Clinical trials are currently attempting to exploit these activities in the treatment of human disease, including cachectic conditions associated with CHF, cancer and COPD, anorexia, ageing and end-stage renal disease. In an animal experiment, favourable effects of ghrelin on burn-induced cachexia were also suggested [57•]. A large number of studies have attempted to elucidate the various activities of ghrelin [58]. A number of these studies may lend support to the development of novel clinical applications for ghrelin in the treatment of

various disorders, particularly cachexia, anorexia and ageing.

Acknowledgements

Research in the authors' laboratory was supported in part by funds from the Ministry of Education, Science, Culture, Sports and Technology of Japan, the Ministry of Health, Labor, and Welfare of Japan, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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First published June 26, 2007; doi:10.1152/ajpendo.00681.2006.

Effects of ghrelin administration on decreased growth hormone status in obese animals

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Submitted 13 December 2006; accepted in final form 22 June 2007

Iwakura H, Akamizu T, Ariyasu H, Irako T, Hosoda K, Nakao K, Kangawa K. Effects of ghrelin administration on decreased growth hormone status in obese animals. *Am J Physiol Endocrinol Metab* 293: E819–E825, 2007. First published June 26, 2007; doi:10.1152/ajpendo.00681.2006.—Obesity is characterized by markedly decreased ghrelin and growth hormone (GH) secretion. Ghrelin is a GH-stimulating, stomach-derived peptide that also has orexigenic action. Ghrelin supplement may restore decreased GH secretion in obesity, but it may worsen obesity by its orexigenic action. To reveal effects of ghrelin administration on obese animals, we first examined acute GH and orexigenic responses to ghrelin in three different obese and/or diabetic mouse models: *db/db* mice, mice on a high-fat diet (HFD mice), and Akita mice for comparison. GH responses to ghrelin were significantly suppressed in *db/db*, HFD, and Akita mice. Food intake of *db/db* and Akita mice were basally higher, and further stimulation of food intake by ghrelin was suppressed. Pituitary GH secretagogue receptor mRNA levels in *db/db* and HFD mice were significantly decreased, which may partly contribute to decreased GH response to ghrelin in these mice. In Akita mice for comparison, decreased hypothalamic GH-releasing hormone (GHRH) mRNA levels may be responsible for decreased GH response, since maximum GH response to ghrelin needs GHRH. When ghrelin was injected into HFD mice with GHRH coadministrated, GH responses to ghrelin were significantly emphasized. HFD mice injected with low-dose ghrelin and GHRH for 10 days did not show weight gain. These results indicate that low-dose ghrelin and GHRH treatment may restore decreased GH secretion in obesity without worsening obesity.

growth hormone secretagogue receptor; obesity; diabetes

IN HUMANS, OBESITY IS CHARACTERIZED by markedly decreased growth hormone (GH) production and secretion (3, 26). GH stimulates lipolysis and increases lean body mass, which may help to combat obesity. Decreased GH secretion in the context of obesity may promote additional fat deposition and promote weight gain (10). GH, however, does contribute to insulin resistance, which could worsen diabetes (7).

Ghrelin is a 28-amino acid peptide with unique acylation modification, which is essential for its biological action (14). Ghrelin was originally identified in the rat stomach as an endogenous ligand for an orphan receptor, which so far has been called GH secretagogue receptor (GHS-R) (14). Ghrelin is involved in a wide variety of functions, including regulation of GH release, gastric acid secretion, gastric motility, blood pressure, and cardiac output (4, 8, 18, 19, 23, 28). Ghrelin also has several metabolic functions, including orexigenic action (20, 22), reduction of insulin (5), and control of energy expenditure (24), which are all involved in the pathophysiology of adiposity or diabetes.

Plasma ghrelin level is suppressed in obesity (25), which may compensate for increased body weight by reducing its orexigenic activity, whereas low plasma ghrelin level may contribute to decreased GH secretion in obesity. Furthermore, Poykko et al. (21) reported that low plasma ghrelin level is associated with insulin resistance and incidence of type 2 diabetes.

To elucidate whether ghrelin supplementation can restore decreased GH secretion in obesity, we first determined acute GH and orexigenic responses to ghrelin in three different obese and/or diabetic mice models: *db/db* mice (a genetically obese mouse model with diabetes), mice on a high-fat diet (HFD; a diet-induced obese mouse model with moderate glucose intolerance), and Akita mice for comparison (an insulin-deprived diabetic nonobese mouse model) (29). Then, we determined how the ghrelin-GH system is modulated in the pituitaries and hypothalamuses of these animals. Last, we examined the effect of chronic ghrelin and GH-releasing hormone (GHRH) administration on diet-induced obesity.

MATERIALS AND METHODS

Experimental animals. Eight-week-old male *db/db* and control mice (misty) were purchased from CLEA Japan, (Tokyo, Japan). As a diet-induced model of obesity, 5-wk-old male C57BL/6J mice, purchased from Japan SLC (Shizuoka, Japan), were maintained on a HFD of 60% fat/kcal (Research Diets, New Brunswick, NJ) for 20 wk. Those maintained on a standard diet were used as control mice for HFD mice. Eight-week-old male Akita mice and C57BL/6J control mice were purchased from Japan SLC. Animals were maintained on standard rat food (CE-2, 352 kcal/100 g; CLEA Japan) with a 12:12-h light-dark cycle unless otherwise indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Acute GH response to ghrelin and GHRH. Rat ghrelin (40, 120, and 360 μg/kg; Peptide Institute, Osaka, Japan), human GHRH (60 μg/kg; Mecasermin, Astellas Pharma, Tokyo, Japan), or saline was injected subcutaneously into mice on an ad libitum feeding schedule. Blood was collected from retroorbital veins 15 or 30 min after injection. Serum was isolated by centrifugation and stored at −20°C until assayed.

Measurements of hormones and free fatty acid levels. Serum GH levels were determined by rat growth hormone EIA kit (SPI bio,

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Table 1. *Basal profiles of db/db mice on HFD and Akita mice*

	Con	*db/db*	Con	HFD	Con	Akita
Weight, g	23.1±0.25	42.3±0.20†	33.3±1.6	50.3±0.9†	24.6±0.5	23.1±0.5
Blood glucose, mg/dl	100.7±6.0	328.0±10.9†	05.5±5.5	125.1±4.1†	125.9±4.2	475.3±16.8†
Insulin, ng/ml	1.35±0.5	25.9±5.7†	3.6±1.1	28.0±6.9†	1.42±0.06	0.25±0.00†
IGF-I, ng/ml	659.9±19.9	764.1±41.5*	301.8±21.6	426.6±20.0†	416.4±21.4	415.8±17.9
FFA, mEq/l	0.78±0.04	1.68±0.11†	0.26±0.10	1.18±0.04	1.20±0.01	1.24±0.05
Ghrelin, fmol/ml	98.4±5.6	34.7±8.6†	164.9±7.5	67.2±10.6†	113.4±11.8	182.7±18.1†

Values are means ± SE. Con, control mice; HFD, mice on a high-fat diet; FFA, free fatty acids. *$P < 0.05$; †$P < 0.01$ compared with control mice or mice on a standard diet; $n = 7$.

Massy Cedes, France). Measurement of serum insulin concentrations was performed by ELISA using an ultrasensitive rat insulin kit (Morinaga, Yokohama, Japan). Serum insulin-like growth factor I (IGF-I) levels were measured using a mouse/rat IGF-I EIA kit (Diagnostic Systems Laboratories, Webster, TX). Serum free fatty acid (FFA) levels were measured by NEFA C test (Wako Pure Chemical Industries, Osaka, Japan).

Measurements of plasma ghrelin concentrations. Measurement of plasma ghrelin levels was performed as reported previously (12). Briefly, blood was drawn from the retroorbital vein after an overnight fast and then immediately transferred to chilled siliconized glass tubes containing Na_2EDTA (1 mg/ml) and aprotinin (1,000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan) and centrifuged at 4°C. Immediately after the plasma was separated, hydrochloric acid was added to samples at final concentration of 0.1 N. Plasma was immediately frozen and stored at −80°C until assay. Plasma ghrelin concentrations were determined using an active ghrelin ELISA kit that recognizes *n*-octanoylated ghrelin (Mitsubishi Kagaku Iatron, Tokyo, Japan) (1).

Real-time quantitative RT-PCR. Total RNA was extracted from the pituitary and hypothalamus using a Sepasol RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription (RT) was performed in the presence of random hexamers with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), using the following primers and TaqMan probes: mouse GH sense, 5′-AAGAGTTCGAGCGTGCCTACA-3′, and antisense, 5′-GAAGCAATTCCATGTCGGTTC-3′, with the TaqMan probe, 5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′; mouse GHRH receptor (GHRH-R) sense, 5′-GCCCTTGGAACTGTTAACCA-3′, and antisense, 5′-GCAACCAGGATGGCAATAGC-3′, with the TaqMan probe, 5′-AGCATCTCCATTGTAGCCCTCTGCGTG-3′; mouse GHS-R sense, 5′-CACCAACCTCTACCTATCCAGCAT-3′, and antisense, 5′-CTGACAAACTGGAAGAGTTTGCA-3′, with the TaqMan probe, 5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′; mouse ghrelin sense, 5′-GCATGCTCTGGATGGACATG-3′, and antisense, 5′-TGGTGGCTTCTTGGATTCCT-3′, with the TaqMan probe, 5′-AGCCCAGAGCACCAGAAAGCCCA-3′; mouse somatostatin receptor (SSTR)2 sense, 5′-GGTCAAGGCAGACAATTCACAA -3′, and antisense, 5′-GTGTTAGCACACATACACACAGGACTT -3′, with the TaqMan probe, 5′-CGGCAGAAACCGGAAAAACCAAAACTAAAT -3′; mouse SSTR5 sense, 5′-CGCTGCCTGACCGCTAAGTA-3′, and antisense, 5′-GCTCACAGAGGTTGGCTCACA-3′, with the TaqMan probe, 5′-CTGCACAGGAGAGGTCTCCACGGCT-3′; mouse GHRH sense, 5′-AGGATGCAGCGACACGTAGA-3′, and antisense, 5′-TCTCCCCTTGCTTGTTCATGA-3′, with the TaqMan probe, 5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′. The mRNA expression in each gene was normalized to that of 18s ribosomal RNA.

Chronic administration of ghrelin and GHRH. Mice on a HFD (HFD mice) or a standard diet (control mice) for 20 wk were injected with 40 μg/kg ghrelin and 60 μg/kg GHRH twice daily for 10 days. Before and after treatment, blood samples were collected and body weights measured. Fat body mass and lean body mass of mice were measured by Latheta LTC-100 (Aloka, Tokyo, Japan) under pentobarbital anesthesia.

Statistical analysis. All values were expressed as means ± SE. The statistical significance of the differences in mean values was assessed by two-way ANOVA or Student's *t*-test as appropriate.

RESULTS

Basal profiles of *db/db*, HFD, and Akita mice are listed in Table 1. *Db/db* and HFD mice showed significantly higher weights, blood glucose, serum insulin, and serum IGF-I levels and significantly lower plasma ghrelin levels than those seen in control mice (Table 1), although the elevation of blood glucose was less severe in HFD mice. Although serum FFA levels of *db/db* mice were significantly higher than those of control mice, those of HFD mice were comparable with those of control mice (Table 1). Although Akita mice demonstrated significantly higher blood glucose levels as either *db/db* mice or HFD mice, Akita mice displayed significantly lower body weights and serum insulin levels and higher plasma ghrelin levels than those seen in control mice (Table 1).

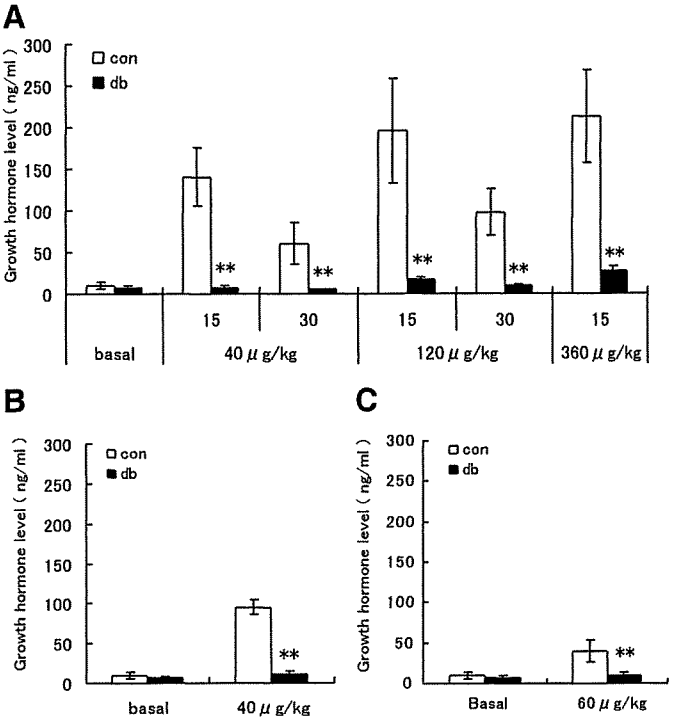

Fig. 1. Growth hormone (GH) responses to ghrelin in *db/db* mice. *A*: serum GH levels 15 or 30 min after sc injection of ghrelin into *db/db* (db) or control (con) mice. *B*: serum GH levels 15 min after iv ghrelin injection into db or con mice. *C*: serum GH levels 15 min after sc injection of GH-releasing hormone (GHRH). **$P < 0.01$.

We first examined acute GH responses to ghrelin in *db/db*, HFD, and Akita mice. GH responses to ghrelin in *db/db* mice were markedly lower than those observed in control mice at any dose (40, 120, or 360 μg/kg; Fig. 1*A*). Thirty minutes after ghrelin injection (40 or 120 μg/kg) of *db/db* mice, serum GH levels tended to be even lower than those at 15 min (Fig. 1*A*), indicating that low GH levels at 15 min were not due to delayed response. GH responses at 15 min after intravenous injection of ghrelin were also decreased in *db/db* mice (Fig. 1*B*), indicating that the disturbed GH responses observed in *db/db* mice were not due to the malabsorption of ghrelin caused by fat deposition at the subcutaneous injection site. GH responses to GHRH (60 μg/kg) were also decreased in *db/db* mice (Fig. 1*C*). As in *db/db* mice, GH levels at 15 min after subcutaneous ghrelin injection (40, 120, and 360 μg/kg) in HFD mice were significantly lower than those seen in control mice (Fig. 2*A*). GH responses to GHRH (60 μg/kg) also tended to be decreased (Fig. 2*B*). Although GH levels in Akita mice were not significantly different from those in control mice measured at 15 min after 40 μg/kg sc injection of ghrelin, those measured after a higher dose of ghrelin (120 and 360 μg/kg) were significantly lower than those in control mice (Fig. 3*A*). GH responses to GHRH (60 μg/kg) also tended to be decreased in Akita mice (Fig. 3*B*).

We then measured 1-h food intake stimulated by ghrelin in *db/db* and Akita mice. In control mice, a 40 μg/kg sc ghrelin injection evoked about threefold greater food intake than that

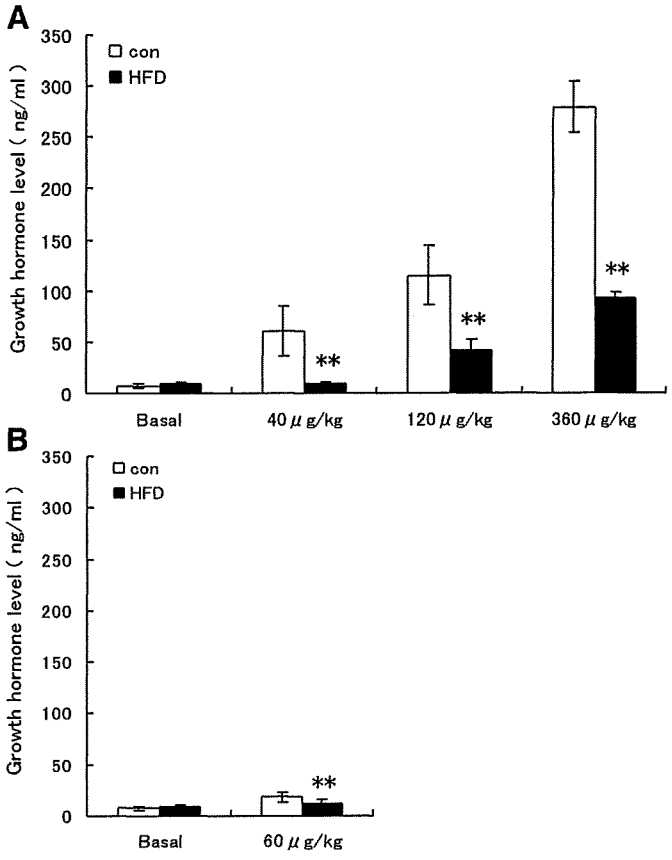

Fig. 2. GH responses to ghrelin in a diet-induced obesity mouse model. *A*: serum GH levels 15 min after sc injection of ghrelin into mice on a high-fat diet (HFD) or con mice. *B*: serum GH levels 15 min after GHRH sc injection. $^{**}P < 0.01$ compared with controls; $n = 7$.

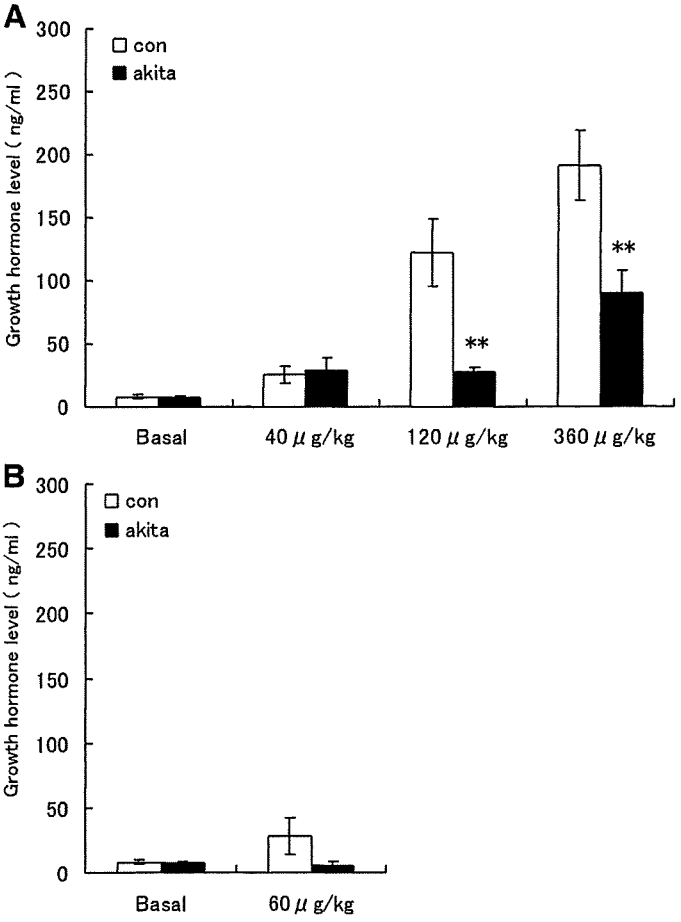

Fig. 3. GH responses to ghrelin in Akita mice. *A*: serum GH levels 15 min after sc injection of ghrelin into Akita or con mice. *B*: serum GH levels 15 min after sc injection of GHRH. $^{**}P < 0.01$ compared with control; $n = 7$.

induced by saline injection (saline vs. ghrelin: 0.09 ± 0.04 vs. 0.28 ± 0.01 g, $P < 0.05$, $n = 7$; Fig. 4*A*). Ghrelin stimulated additional food intake in a dose-dependent manner in control mice, as demonstrated by the ratio of food intake evoked by ghrelin to that induced by saline (Fig. 4*A*). In *db/db* mice, basal food intake was higher than that of control mice (0.22 ± 0.05 vs. 0.07 ± 0.02 g, $P < 0.05$, $n = 7$). Although sc injection of 40 μg/kg ghrelin into *db/db* mice did not stimulate food intake significantly (saline vs. ghrelin: 0.30 ± 0.11 vs. 0.18 ± 0.04 g, $P = 0.30$, $n = 7$), higher doses of ghrelin (360 μg/kg), however, were able to stimulate food intake (saline vs. ghrelin: 0.14 ± 0.08 vs. 0.39 ± 0.04 g, $P < 0.05$, $n = 7$). Although higher ghrelin stimulated food intake in *db/db* mice, the extent of stimulation as demonstrated by the ratio of ghrelin-induced food intake (40 and 360 μg/kg) to that by saline was significantly smaller than that in control mice (Fig. 4*A*). In Akita mice, basal food intake was higher than that of control mice (0.28 ± 0.01 vs. 0.15 ± 0.02 g, $P < 0.05$, $n = 7$), and no further stimulation of food intake by ghrelin was observed (Fig. 4*B*).

We then measured the mRNA expression of ghrelin-GH system in pituitaries and hypothalamuses of *db/db*, HFD, and Akita mice (Fig. 5). Pituitary mRNA levels of GHS-R were significantly lower in *db/db* and HFD mice, whereas those in Akita mice were significantly higher compared with their

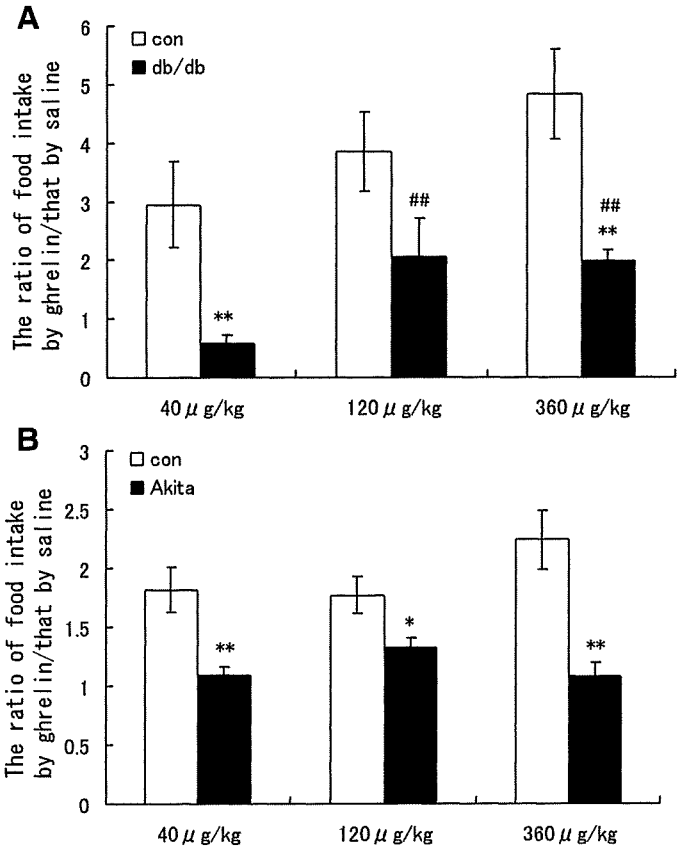

Fig. 4. Food intake stimulated by sc ghrelin injection. *A*: the ratio of 1-h food intake after sc ghrelin injection to that observed after sc saline injection in db and con mice. *B*: the ratio of the food intake observed for 1 h after sc ghrelin injection to that seen after saline injection for Akita and con mice. $*P < 0.05$; $**P < 0.01$ compared with control; $\#\#P < 0.01$ compared with 40 μg/kg; $n = 7$.

control mice (Fig. 5*A*). Pituitary mRNA levels of ghrelin were significantly lower in *db/db* mice, whereas those in Akita mice were significantly higher (Fig. 5*A*). Pituitary mRNA levels of GH were significantly lower in *db/db* and HFD mice and tended to be lower in Akita mice (Fig. 5*A*). Pituitary mRNA levels of SSTR2 were significantly higher in *db/db* mice, whereas they were not significantly changed in HFD and Akita mice (Fig. 5*A*). Pituitary mRNA levels of SSTR5 were significantly lower in Akita mice, whereas those levels were not significantly changed in *db/db* and HFD mice (Fig. 5*A*). There were no significant changes in the expression levels of GHRH-R in pituitaries of these mice (Fig. 5*A*). In hypothalamus, GHS-R and GHRH mRNA levels were significantly higher and lower, respectively, in Akita mice (Fig. 5*B*). Ghrelin mRNA levels were significantly lower in hypothalamus of HFD mice (Fig. 5*B*).

Finally, we examined the effect of chronic ghrelin injection to HFD mice. To maximize GH-stimulating activity of ghrelin and to minimize orexigenic action of ghrelin, we first examined GH responses to low-dose ghrelin with GHRH coadministration in HFD mice. Acute GH responses to ghrelin were significantly potentiated by coadministration of GHRH even at the lowest dose in HFD mice (Fig. 6*A*). By 10 days of twice daily injections of saline or ghrelin and GHRH, both control and HFD mice lose weight by ~6–8%. This weight reduction might be due to stress of twice daily injection, which is usually covered by growth in younger mice. Control mice treated with ghrelin and GHRH tended to take in more food than those with saline (Fig. 6*C*). Fat masses were more preserved in the ghrelin- and GHRH-treated groups than in the saline-treated group in control mice (Fig. 6, *B*, *D*, and *E*), although percent body weight and percent lean body mass changes were comparable between the saline-treated and the ghrelin- and GHRH-treated group in control mice. In HFD mice, food intake, percent body weight change, and percent lean body mass change were comparable between the saline-treated group and the ghrelin- and GHRH-treated groups (Fig. 6, *B*, *D*, and *E*). In contrast to control mice, fat mass tended to even be decreased in the ghrelin and GHRH group than in the saline-treated group in HFD mice (Fig. 6*D*). In both control and HFD mice, blood glucose, serum insulin, and serum IGF-I levels of the ghrelin- and GHRH-treated group were not significantly different from those of the saline-treated group (Fig. 6, *F*, *G*, and *H*).

DISCUSSION

We have demonstrated that GH responses to ghrelin are decreased in both genetic and diet-induced mouse models of obesity. Recently, Luque and Kineman (15) reported that plasma GH levels acquired by random sampling without stimulation in *ob/ob* mice and HFD mice tended to be lower than

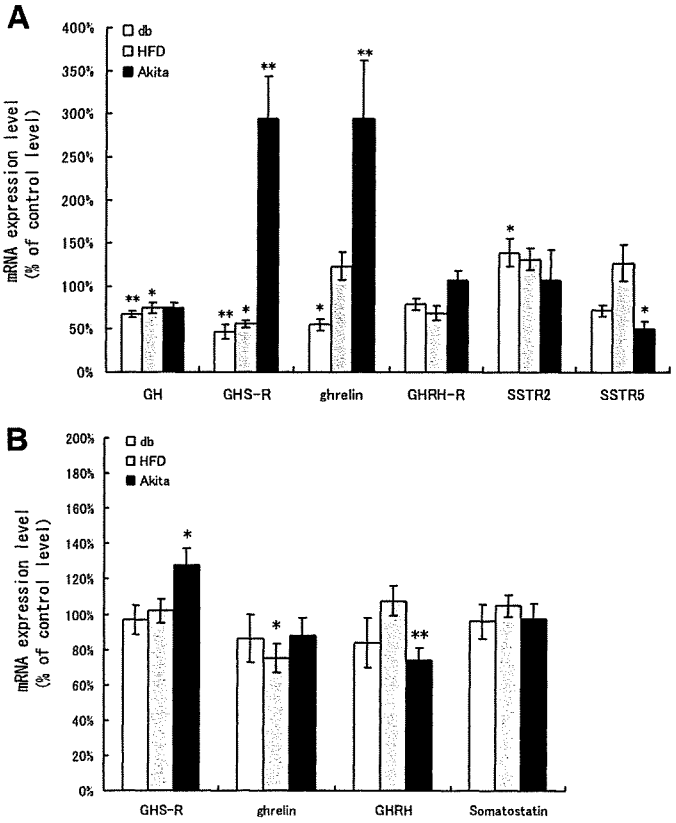

Fig. 5. The mRNA expression levels in the pituitaries or hypothalamuses of db mice, mice on a HFD, and Akita mice. *A*: the mRNA expression levels of GH, GH secretagogue receptor (GHS-R), ghrelin, GH-releasing hormone receptor (GHRH-R), somatostatin receptor (SSTR)2, and SSTR5 in the pituitaries of db, HFD, and Akita mice. *B*: the mRNA expression levels of GHS-R, ghrelin, GHRH, and somatostatin in the hypothalamuses of db, HFD, and Akita mice. Data were presented as % of the level seen in control mice. $*P < 0.05$; $**P < 0.01$ compared with control; $n = 14$.

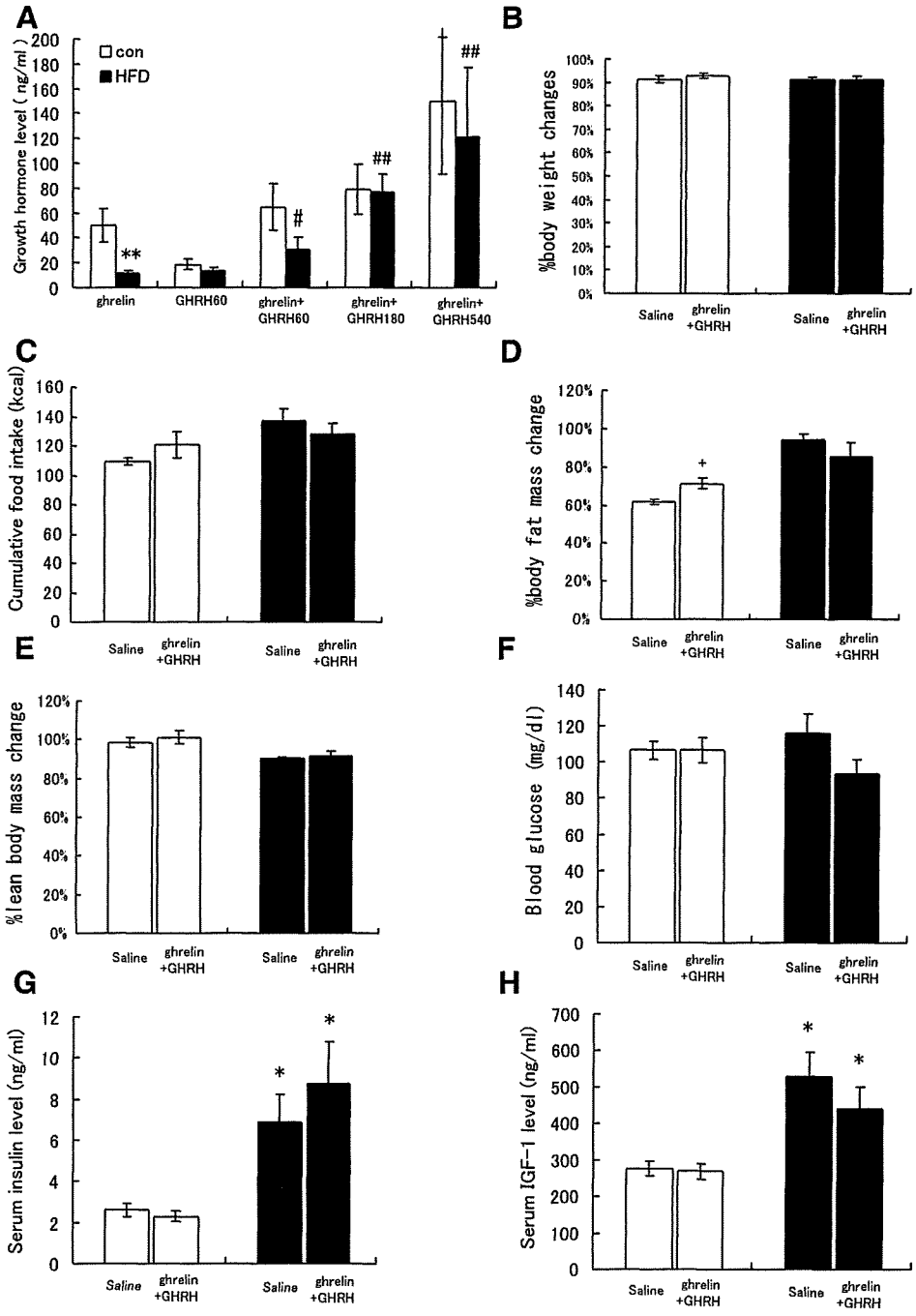

Fig. 6. Chronic treatment of ghrelin and GHRH on mice on a HFD. *A*: serum GH levels 15 or 30 min after sc injection of 40 μg/kg ghrelin or 60 μg/kg GHRH or ghrelin and GHRH (60, 180, 540 μg/kg) into HFD or con mice. %Body weight (*B*), fat mass (*D*), and lean body mass (*E*) changes before and after treatment of 40 μg/kg ghrelin and 60 μg/kg GHRH or saline for 10 days in control mice (open bars) or mice on a HFD (filled bars). *C*: cumulative food intake for 10 days. Blood glucose (*F*), serum insulin level (*G*), and serum IGF-I level (*H*) after 10 days administration of ghrelin and GHRH. $*P < 0.05$; $**P < 0.01$ compared with control; $\#P < 0.05$; $\#\#P < 0.01$ compared with ghrelin; $+P < 0.05$ compared with saline; $n = 7$.

those seen in control mice. Because GH secretion is pulsatile, it is difficult to compare GH values obtained by random sampling in the absence of stimulation. For this reason, we could not detect any significant difference in basal GH values between *db/db* or HFD and control mice. Following ghrelin or GHRH stimulation, however, we clearly observed a severe impairment in GH secretions by obese mice. Alvarez-Castro et al. (2) previously reported that GH responses to ghrelin were decreased in obese human subjects compared with those seen in normal controls; although reduced, these responses to ghrelin were greater in magnitude than those observed following GHRH treatment in obese human subjects. Our observations in mice were consistent with these data obtained in humans, which verify the use of *db/db* and HFD mice as experimental animal models for ghrelin treatment for obesity.

We also demonstrated that GH responses to ghrelin are decreased in Akita mice. As far as we know, this is the first report on the GH responses to ghrelin in insulin-deprived mice. In humans with insulin-deprived diabetes, it is well known (11) that basal GH is elevated and that GH response to provocative tests, including GHRH or GHS administration, is exaggerated. Thus discrepancy between human and mouse GH response to ghrelin in insulin-deprived status exist.

We demonstrated that GHS-R mRNA levels were decreased in the pituitaries of *db/db* and HFD mice. Ghrelin does stimulate GH release from rat pituitary in vitro (14), but maximal

response of GH to ghrelin requires the existence of GHRH (9). Kamegai et al. (13) reported that pituitary ghrelin regulates GH secretion by modulating pituitary response to GHRH. The decreased expression of GHS-R in pituitary in *db/db* and HFD mice might contribute to suppressed GH response to ghrelin by attenuating the pituitary response to GHRH. Of course, decreased mRNA levels of GH in pituitary or, as reported in human (6, 16), elevated serum IGF-I or FFA levels in these mice might also contribute to suppressed GH responses.

Although GH responses to ghrelin were also decreased in Akita mice, the pituitary mRNA levels of GHS-R were significantly higher than those seen in control mice, indicating that pituitary GHS-R did not contribute to decreased GH responses. GHRH mRNA expression levels in hypothalamus of Akita mice were significantly lower compared with those of control mice. This reduction of GHRH mRNA levels may be responsible for decreased GH responses to ghrelin in Akita mice. Ghrelin stimulates GHRH secretion from the hypothalamus (27). And recently, Mano-Otagiri et al. (17) reported that GHS-R signaling upregulates hypothalamic GHRH expression. Although plasma ghrelin levels were significantly higher in Akita mice than those displayed by control mice, GHRH mRNA expression levels in hypothalamus of Akita mice were significantly decreased. In addition, the food intake of Akita mice was significantly elevated at baseline and was not stimulated by ghrelin any further. These results indicate the existence of ghrelin unresponsiveness in postreceptor level in hypothalamus.

In the chronic treatment experiment, ghrelin and GHRH treatment for 10 days tended to stimulate food intake and showed fat-sparing effect in control mice. In contrast, HFD mice injected with ghrelin and GHRH tended to decrease more fat mass compared with those treated with saline, which may be due to restored GH secretion and suppressed orexigenic response to ghrelin. In this setting, blood glucose and serum insulin levels did not change by ghrelin and GHRH treatment in HFD mice. This may be explained by the fact that the change in fat mass was only subtle and that lean body mass did not change by ghrelin and GHRH treatment. These results indicate that low-dose ghrelin and GHRH supplementation at least do not worsen obesity and metabolic status and that it may at least partially restore suppressed GH secretion.

In the current experiment, IGF-I levels were higher in HFD mice after chronic treatment of ghrelin and GHRH. Since IGF-I levels of the saline-treated group of HFD mice were also higher than those of control mice, this elevation seems to reflect nutritional status between HFD and control mice.

In conclusion, we demonstrated that acute GH responses to ghrelin were suppressed in both genetic and diet-induced mouse models of obesity. The decreased pituitary levels of GHS-R mRNA may contribute to suppression of GH response. We also demonstrated that acute GH responses to ghrelin were suppressed in Akita mice, an insulin-deprived diabetic mouse model. Decreased GHRH mRNA levels in hypothalamus and the lack of stimulation of food intake by ghrelin indicate the involvement of hypothalamus in the mechanism of suppressed GH response to ghrelin in Akita mice. These results indicate that suppressed GH response to ghrelin has a different mechanism in obese and insulin-resistant mice and insulin-deprived diabetic animals. In addition, HFD mice injected with ghrelin and GHRH showed potentiated GH responses. Chronic treatment of low-dose ghrelin and GHRH did not promote fat deposition in HFD mice. These results indicate that low-dose ghrelin and GHRH administration at least does not worsen obesity and that it may restore suppressed GH secretion.

ACKNOWLEDGMENTS

We thank Hitomi Hiratani, Chieko Ishimoto, Naoko Takehisa, Kozue Fukuda, and Chinami Shiraiwa for excellent technical assistance.

GRANTS

This study was supported by funds from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Ministry of Health, Labour, and Welfare of Japan.

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