administration on the relationship between plasma ghrelin and the somatotropic axis in rats. We investigated plasma ghrelin concentrations after a single LPS administration and after repeated LPS administration, using two kinds of RIAs. In addition, we examined the therapeutic potential of the antiwasting effect of ghrelin using rats repeatedly injected with LPS.

Materials and Methods

Animals

Seven-week-old male Wister rats (200–250 g) were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a temperature-, humidity-, and light-controlled room (12-h light/12-h dark cycle, lights on at 0800 h) and allowed free access to water and standard rat food (CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan), unless otherwise indicated. Rats were individually housed in Experiments 2 and 3. Animals were weighed, and food intake was measured every morning. One-day food intake was measured by subtracting the amount of uneaten food on each day from that provided the previous day. Food spillage into the cage was assessed and found to be negligible. All experimental procedures were approved by the Kyoto University Graduate School of Medicine committee on animal research.

Experiment 1: single administration of LPS

Rats were injected ip with 0 or 10 mg/kg LPS (serotype O26:B6; Sigma-Aldrich Corp., St. Louis, MO) in 300 μl saline at 0800 h. The LPS dose used in the present study was sublethal; the 50% lethal dose was approximately 50 mg/kg body weight. For determination of plasma levels of ghrelin and serum concentrations of GH and IGF-I, animals were anesthetized with pentobarbital, and trunk blood was collected at 3, 6, 12, and 24 h post injection. Food was withdrawn after endotoxin injection because LPS-induced anorexia may change the plasma ghrelin concentration. To establish a dose dependency for LPS-induced changes in energy balance, animals were injected with 0, 0.1, 1, or 10 mg/kg LPS, and trunk blood was collected 12 h post injection.

Experiment 2: repeated administration of LPS

Rats were injected ip with 0 or 1 mg/kg LPS in 300 μl saline once daily at 0800 h for 2 or 5 d. Pair-fed (PF) rats were injected with saline and given the same amount of food as that consumed by LPS-treated rats on the previous day. Food was withdrawn after the final LPS injection. Twelve hours later, animals were anesthetized with pentobarbital, and trunk blood was collected and stored until hormone assays were carried out.

Experiment 3: ghrelin administration in rats with LPS-induced wasting syndrome

Rats were divided into four groups: control (n = 9), ghrelin (n = 8), LPS (n = 8), and LPS plus ghrelin (n = 8). Then 0 or 1 mg/kg LPS in 300 μl saline was injected ip once daily at 0800 h for 5 d. Fourteen hours after the first LPS injection, 0 or 10 nmol/rat ghrelin in 300 μl saline were injected sc twice daily at 1000 and 2200 h for 5 d. Ghrelin (10 nmol/rat) was chosen, as this dose was previously demonstrated to increase food intake 0–2 h after peripheral administration (24). Rat ghrelin was obtained from Peptide Institute, Inc. (Osaka, Japan). After the last weight measurement, animals were anesthetized with pentobarbital, trunk blood was collected, and the liver, spleen, adrenal gland, and testicular fat pad were dissected and weighed.

Blood sampling and assay for plasma ghrelin

Plasma samples were prepared as previously described (7). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA and aprotinin and centrifuged at 4 C. Plasma was acidified with hydrogen chloride for a final concentration of 0.1 N immediately after separation and were stored at −80 C until use. The plasma ghrelin concentration was measured by an RIA specific for ghrelin as described previously: C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin (17, 18). In brief, 1 ml of the prepared plasma sample was applied to a Sep-Pak C_{18} cartridge (Waters Corp., Milford, MA). After washing, the cartridge was eluted with 3.0 ml 60% CH_3CN/0.1% trifluoroacetic acid. The eluate was subjected to RIA. The minimal detectable quantities by C-RIA and N-RIA were 5.0 and 0.5 fmol/tube, respectively.

Other biochemical measurements

Serum GH and leptin were measured with enzyme immunoassay kits (Biotrak EIA, Amersham Pharmacia Biotech, Arlington Heights, IL). Serum IGF-I was determined with an enzyme immunoassay kit (Active Rat IGF-I EIA, Diagnostic Systems Laboratories, Inc.,).

Statistical analysis

All values are presented as the mean ± SEM. Comparisons between groups were performed using the unpaired *t* test. For comparisons among several groups, statistical significance was determined using one-way ANOVA with the *post hoc* least significant difference test. $P < 0.05$ was considered significant.

Results

Single LPS administration

As LPS administration induces anorexia, the experiment was performed under fasting conditions after LPS injection. Therefore, plasma ghrelin levels in saline-injected control rats gradually increased after fasting, whereas plasma ghrelin levels were suppressed 6 and 12 h after the administration of 10 mg/kg LPS (Fig. 1A). Plasma ghrelin levels measured by C-RIA remained at 70.0% ($P < 0.05$) and 63.8% ($P < 0.01$) of control levels, respectively, and those measured by N-RIA were 83.2% (P = NS) and 66.8% ($P < 0.05$) of control levels, respectively. Twenty-four hours after LPS ad-

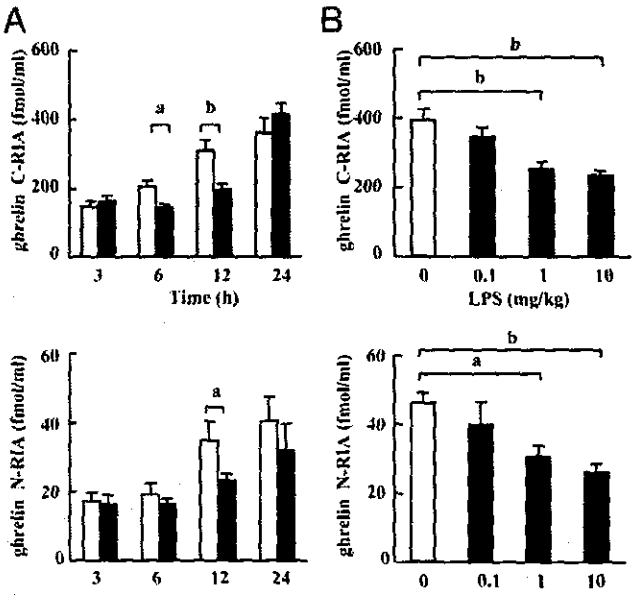

FIG. 1. The effect of a single LPS injection on plasma ghrelin levels. At 0800 h, fasting was started, and rats were injected ip with saline (□) or LPS (■). A, Changes in plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*) after the administration of 10 mg/kg LPS. B, Changes in plasma ghrelin levels 12 h after the injection of various doses of LPS. Values are the mean ± SEM (n = 6 for each group). a, $P < 0.05$; b, $P < 0.01$ (*vs.* saline).

ministration, plasma ghrelin levels returned to control levels. Further, suppression of plasma ghrelin levels 12 h after LPS administration appeared to be dose dependent (Fig. 1B). The plasma ghrelin levels determined by C-RIA after 0.1, 1, and 10 mg/kg LPS administration were reduced to 87.5% (P = NS), 63.9% ($P < 0.01$), and 59.5% ($P < 0.01$) of control levels, respectively. Similarly, plasma ghrelin levels determined by N-RIA after 0.1, 1, and 10 mg/kg LPS administration were reduced to 86.1% (P = NS), 65.8% ($P < 0.05$), and 56.4% ($P < 0.01$) of control levels, respectively.

Serum GH levels were significantly reduced 3 h after LPS injection by 45.3% relative to levels in control rats, but thereafter GH concentrations recovered (Fig. 2A). IGF-I levels were significantly reduced to 72.4%, 85.9%, and 79.1% compared with control rats at 6, 12, and 24 h after LPS administration, respectively (Fig. 2B).

Repeated LPS administration

As chronic LPS treatments have been reported to change the activity levels of the somatotropic axis (23), we examined plasma ghrelin levels on d 2 and 5. Body weight gain was significantly reduced in LPS-treated and PF rats on d 2 (LPS-treated, −4.4 ± 1.6 g; PF, −3.2 ± 1.2 g; saline-treated, 17.2 ± 1.7 g; $P < 0.01$ *vs.* saline for both) and d 5 (LPS-treated, 21.3 ± 1.7 g; PF, 16.4 ± 2.3 g; saline-treated, 38.3 ± 1.8 g; $P < 0.01$ *vs.* saline for both). On d 2 plasma ghrelin levels measured by C-RIA in LPS-treated rats were 1.9-fold higher than those in control rats and 1.4-fold higher than those in PF rats. Plasma ghrelin levels determined by N-RIA in LPS-treated rats were 3.4-fold higher than those in control rats and 2.0-fold higher than those in PF rats (Fig. 3A). On d 5 plasma ghrelin levels measured by C-RIA in LPS-treated rats were higher than those in control or PF rats, although the levels were decreased compared with those on d 2 (d 2, 648.9 ± 50.7; d 5, 495.9 ± 31.6 fmol/ml; $P < 0.05$). Plasma ghrelin levels measured by N-RIA in LPS-treated rats were also higher than those in control rats on d 5, although levels were decreased relative to those on d 2 (d 2, 87.3 ± 10.5; d 5, 52.2 ± 5.1 fmol/ml; $P < 0.01$; Fig. 3A). Although plasma ghrelin levels in PF rats were significantly increased compared with controls, the levels were lower than those in LPS-treated rats (Fig. 3A). In contrast, administration of LPS for 2 and 5 d resulted in decreased serum GH concentrations relative to those in control and PF rats, although it did not significantly reduce serum IGF-I levels (Fig. 3B).

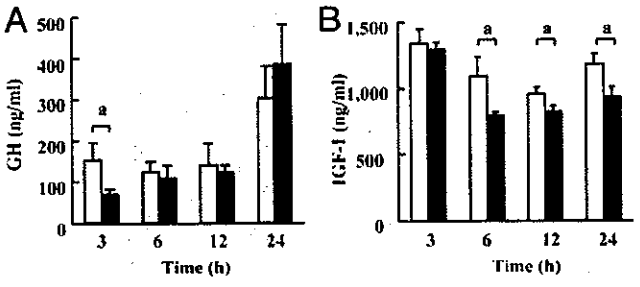

FIG. 2. The effect of a single LPS (10 mg/kg) injection on serum GH (A) and IGF-I (B) concentrations. Values are the mean ± SEM (n = 6 for each group). □, Saline; ■, LPS. a, $P < 0.05$ (*vs.* saline).

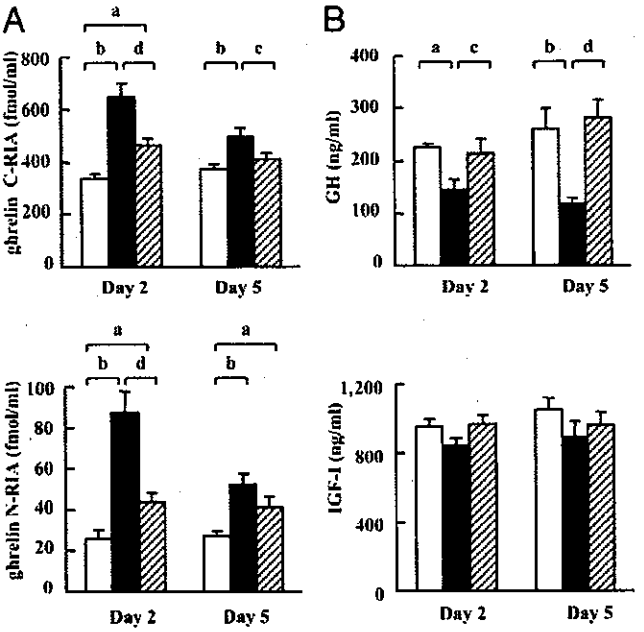

FIG. 3. The effect of repeated LPS injection on plasma ghrelin levels (A), and serum GH and IGF-I concentrations (B). Twelve hours after the final injection, blood was taken, and plasma was separated for the assays indicated. A, Changes in plasma ghrelin levels measured by C-RIA (*upper panel*) and N-RIA (*lower panel*). B, Changes in serum GH (*upper panel*) and IGF-I (*lower panel*) concentrations. Values are the mean ± SEM (n = 9 for each group). a, $P < 0.05$; b, $P < 0.01$ (*vs.* saline). c, $P < 0.05$; d, $P < 0.01$ (*vs.* PF). □, Saline; ■, LPS; ▨, PF.

Ghrelin administration in rats with LPS-induced wasting syndrome

Daily food intake was suppressed on d 1 (LPS, 12.0 ± 0.5 g; control, 20.7 ± 0.9 g; $P < 0.01$), d 2 (LPS, 18.6 ± 0.7 g; control, 22.4 ± 0.6 g; $P < 0.01$), and d 4 (LPS, 21.0 ± 1.1 g; control, 24.3 ± 0.9 g; $P < 0.05$) in rats treated with 1 mg/kg LPS (Fig. 4A). Furthermore, cumulative food intake over 5 d was significantly decreased relative to that in control groups (LPS, 95.5 ± 2.3; control, 112.4 ± 3.0 g/5 d; $P < 0.01$; Fig. 4B). Body weight was decreased on d 1 and began recovering on d 2, but was 51.8% lower than the control value on d 5 (LPS, 18.6 ± 2.1; control, 35.9 ± 2.2 g/5 d; $P < 0.01$; Fig. 4, C and D). To examine the effects of ghrelin on food intake and body weight, we administered saline (control) or 10 nmol/rat ghrelin sc twice daily. Treatment with ghrelin for 5 d significantly increased cumulative food intake relative to that of control groups (ghrelin, 121.3 ± 2.4; control, 112.4 ± 3.0 g/5 d; $P < 0.05$; Fig. 4B). The increased food intake induced by ghrelin was associated with a significant increase in body weight gain (ghrelin, 41.1 ± 1.6; control, 35.9 ± 2.2 g/5 d; $P < 0.05$; Fig. 4D). Although no significant difference was noted with ghrelin treatment in repeated LPS-injected rats in either daily or cumulative food intake, the LPS-induced reduction in body weight on d 5 was significantly attenuated by ghrelin treatment (ghrelin plus LPS, 24.9 ± 1.9; LPS, 18.6 ± 2.1 g/5 d; $P < 0.05$; Fig. 4D).

Spleen weights of LPS-injected rats were significantly increased relative to those of control rats (LPS, 0.81 ± 0.04; control, 0.65 ± 0.02 g; $P < 0.01$), but were not significantly

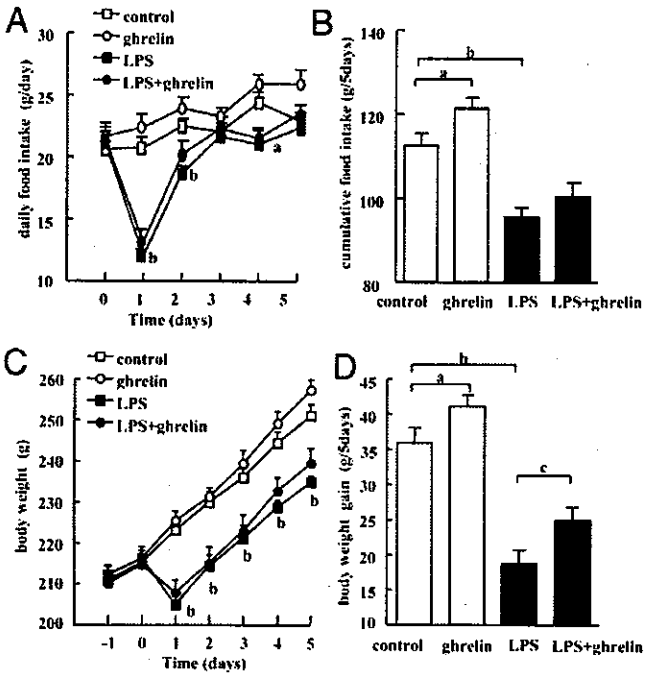

FIG. 4. The effect of ghrelin treatment with repeated LPS injection (1 mg/kg) on daily food intake (A), cumulative food intake (B), body weight (C), and body weight gain 5 d after the first injection (D). Values are the mean ± SEM (n = 8–9 for each group). a, $P < 0.05$; b, $P < 0.01$ (*vs.* control). c, $P < 0.05$ (*vs.* LPS).

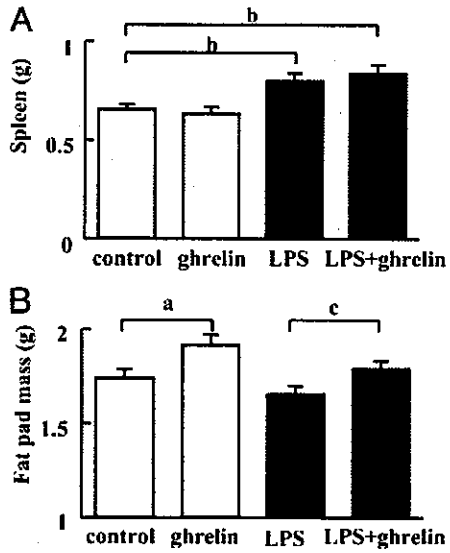

FIG. 5. The effect of ghrelin treatment with repeated LPS injection on spleen (A) and testicular fat pad (B) mass weights. Values are the mean ± SEM (n = 8–9 for each group). a, $P < 0.05$; b, $P < 0.01$ (*vs.* control). c, $P < 0.05$ (*vs.* LPS).

modified by ghrelin treatment (Fig. 5A). White adipose tissue, as assessed by epididymal fat pad mass, was significantly increased in rats treated with ghrelin (ghrelin, 1.91 ± 0.06; control, 1.74 ± 0.05 g; $P < 0.05$) and increased still further in LPS-injected and ghrelin-treated rats (ghrelin plus LPS, 1.79 ± 0.04; LPS, 1.65 ± 0.04 g; $P < 0.05$; Fig. 5B). There was no significant difference in liver and adrenal weights among these groups (data not shown).

Serum leptin concentrations were significantly increased by ghrelin treatment in LPS-injected rats (LPS plus ghrelin, 1.21 ± 0.24; LPS, 0.92 ± 0.32 ng/ml; $P < 0.05$), but were not significantly increased in normal control rats (Fig. 6). Repeated LPS injection decreased serum levels of albumin and hemoglobin and increased platelet counts (Table 1). Treatment with ghrelin caused a modest recovery in hemoglobin concentration from 11.9 ± 0.2 to 12.4 ± 0.2, but did not modify serum albumin levels or platelet counts. There were no significant differences in serum GH (control, 280.6 ± 49.5; ghrelin, 287.5 ± 41.8; LPS, 211.4 ± 18.7; LPS plus ghrelin, 176.6 ± 13.8 ng/ml) or IGF-I (control, 1223.1 ± 86.5; ghrelin, 1248.0 ± 99.7; LPS, 1152.1 ± 44.4; LPS plus ghrelin, 1210.1 ± 67.7 ng/ml) concentrations after ghrelin treatment.

Discussion

This is the first report showing that a single LPS injection suppressed plasma ghrelin levels, wheras repeated LPS injection elevated plasma ghrelin levels. During revision of this manuscript, two independent groups reported that a single LPS injection shifted plasma ghrelin levels of male Sprague-Dawley rats: one group showed that plasma ghrelin levels were decreased 3 h after ip injection of 0.1 mg/kg LPS, and the other showed that levels were increased 24 h after iv injection of 5 mg/kg LPS (25, 26). In this study a single LPS injection suppressed plasma ghrelin levels 6 and 12 h after LPS injection. Maximal reduction was observed 12 h after LPS injection, and ghrelin was reduced in a dose-dependent manner. On the other hand, 3 h after LPS injection serum concentrations of GH were decreased, as were subsequent serum concentrations of IGF-I. Therefore, it is unlikely that the inhibitory effect of a single LPS injection on the somatotropic axis was mediated by the reduction of plasma ghrelin. This supports the recent idea that although ghrelin administration strongly increases GH secretion in animal

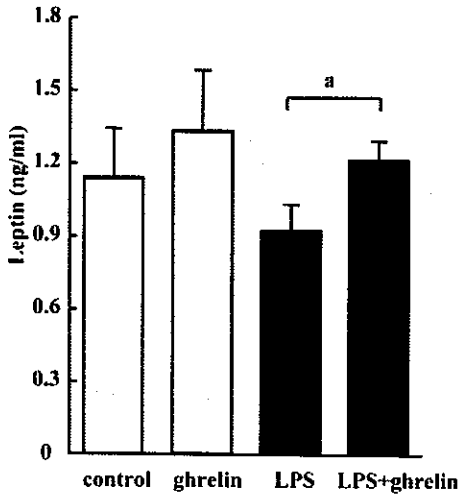

FIG. 6. The effect of ghrelin treatment with repeated LPS injections on serum leptin concentrations. Values are the mean ± SEM (n = 8–9 for each group). a, $P < 0.05$ (*vs.* LPS).

TABLE 1. Effect of ghrelin on biological activities of LPS

	Control	Ghrelin	LPS	LPS + ghrelin
Total protein (g/dl)	4.79 ± 0.10	4.66 ± 0.08	4.60 ± 0.07	4.67 ± 0.08
Albumin (g/dl)	3.46 ± 0.07	3.40 ± 0.10	2.97 ± 0.07[a]	3.08 ± 0.06[a]
Blood urea nitrogen (mg/dl)	19.9 ± 1.2	20.4 ± 0.9	20.7 ± 1.4	19.7 ± 0.9
Creatinine (mg/dl)	0.21 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
Hemoglobin (g/dl)	13.7 ± 0.2	13.4 ± 0.2	11.9 ± 0.2[a]	12.4 ± 0.2[a,b]
Platelet count ($\times 10^3$/mm^3)	101.3 ± 3.8	91.2 ± 4.1	116.1 ± 4.4[c]	113.6 ± 5.8[c]

Values are the mean ± SEM (n = 8–9 for each group).
[a] $P < 0.01$ *vs.* control.
[b] $P < 0.05$ *vs.* LPS.
[c] $P < 0.05$ *vs.* control.

models and humans, a role for ghrelin in endogenous GH secretion is not yet clear (27, 28). The effects of LPS on the neuroendocrine system were suggested to be mediated by cytokines released by LPS-stimulated macrophages and monocytes (29). Indeed, IL-1β decreased gastric ghrelin mRNA expression (15). Alternatively, inhibition of plasma ghrelin levels by a single LPS injection may be mediated by somatostatin. Previous studies reported an increase in somatostatin release induced by acute LPS treatment both *in vivo* and *in vitro* (30, 31), and the suppression of GH secretion by acute endotoxin administration was reversed by antisomatostatin serum (32). In fact, plasma ghrelin levels have been reported to be suppressed by somatostatin (33). As plasma ghrelin levels have been shown to be suppressed by other factors, including increased body mass index and energy surplus (19, 34), insulin (35), glucose (19, 36), and hyperthyroidism (37), further studies are needed to elucidate the mechanism for this reduction.

Soto *et al.* (23) showed that chronic LPS administration (250 μg/kg daily for 8 d) decreased body weight, serum levels of IGF-I, and pituitary GH content and increased hypothalamic somatostatin content. We showed that repeated LPS injection caused body weight loss on d 2 and 5 in rats treated with 1 mg/kg LPS. Furthermore, serum GH concentrations were decreased on d 2 and 5. In contrast, plasma ghrelin levels were elevated after repeated LPS injection. Moreover, as plasma ghrelin levels in LPS-treated rats were even higher than those in PF rats, this increment was not purely induced by anorexia. Fasting (38), anorexia nervosa (38), cancer cachexia (16, 39), and chronic heart disease (14) have been previously reported to cause elevated plasma ghrelin levels. Similarly, elevation of ghrelin levels in rats after repeated LPS injection may reflect a physiological adaptation to negative energy balance.

Prolonged or repeated LPS administration leads to endotoxin tolerance, which is also observed with continuous endotoxin infusion (40, 41). Plasma ghrelin levels on d 5 after repeated LPS injection were lower than those on d 2. This difference in plasma ghrelin levels may be the result of tolerance. The reduced hemoglobin and albumin levels on d 5, however, indicated that the LPS-induced wasting state persisted. Most animal models of sepsis induce high mortality or early recovery and do not mimic the long-lasting catabolic state observed in patients (42). Chronic LPS administration appears to have several advantages as a wasting model. The dose of LPS can be accurately measured to adjust the severity of the condition, and furthermore, the activity of the somatotropic axis is altered after LPS administration (23).

In this study we used two kinds of RIAs, namely C-RIA for the carboxyl terminal and N-RIA for the amino terminal of ghrelin. The levels measured by N-RIA represent the active form of ghrelin with an *n*-octanoyl modification at Ser^3, whereas the levels measured by C-RIA represent total ghrelin, including its inactive form (17). After both single and repeated LPS injections, we could not detect a marked discrepancy between ghrelin levels measured by C-RIA and N-RIA, in agreement with a previous study (19). The effect of glucose injection on plasma ghrelin levels in fasted Sprague Dawley rats was, however, observed more obviously in N-RIA than C-RIA (19). As there may be physiological or pathological conditions in which plasma levels of the total and active forms of ghrelin differ, it is necessary to measure the acylated form of this hormone in various conditions.

We showed that repeated LPS injection induced anorexia, weight loss, and hypoalbuminemia and reduced hemoglobin, characteristic of the wasting syndrome. In this study this model was used to examine the therapeutic potential of the antiwasting effects of ghrelin. We demonstrated that peripheral administration of ghrelin accelerated body weight gain in this model. However, we did not observe increased food intake in LPS-injected rats treated with ghrelin. This discrepancy may be explained by ghrelin-induced metabolic changes, which lead to a more efficient metabolic state, resulting in increased body weight and fat mass (10). Careful examination revealed that ghrelin treatment was effective at increasing adipose tissue weight in wasting rats. Furthermore, we confirmed that plasma leptin concentrations, which were thought to be positively correlated with the quantity of adipose tissue, were increased by ghrelin treatment in these rats. These findings suggest that chronic ghrelin treatment is likely to cause body weight gain and adipogenesis, even in the LPS-induced wasting state.

In the present study repeated LPS injection reduced serum hemoglobin and albumin levels. Ghrelin treatment modestly increased hemoglobin concentrations in LPS-injected rats, but had no effect on serum albumin concentrations. A few studies have reported that GH treatment resulted in increased hemoglobin concentrations in anemic patients with GH deficiency (43, 44). Thus, the increased hemoglobin concentrations induced by ghrelin treatment of LPS-injected rats could be explained by an anabolic effect of ghrelin mediated through GH/IGF-I. Furthermore, some studies showed that GH reduced net protein loss in the critically ill (5, 43, 44), whereas others demonstrated that GH treatment did not reduce protein catabolism (5, 45). This discrepancy suggests

that the dose and timing of GH administration may be important. Further detailed studies are needed to fully establish the role of ghrelin treatment in the wasting syndrome.

The roles of GH and IGF-I in the regulation of immune function have been investigated (46). GH and IGF-I exert independent effects on lymphoid tissue, and the administration of these agents resulted in splenic enlargement. In the present study spleen weights of LPS-injected rats were increased compared with those of control rats, but they were not modified by ghrelin treatment. Furthermore, depending on the experimental conditions, GH can either reduce (47) or increase (4) susceptibility to endotoxin or bacterial challenge in animals. We observed that the biological activity of 10 mg/kg LPS was not enhanced by priming rats with 10 nmol/rat ghrelin twice daily for 5 d (data not shown). This suggests that ghrelin treatment can improve the endotoxin-induced wasting syndrome without changing the sensitivity to endotoxin.

In summary, the present study demonstrates that a single LPS injection suppressed plasma ghrelin levels in rats, whereas repeated LPS injection elevated ghrelin levels. Chronic sc administration of ghrelin increased body weight gain, adipose tissue weight, and plasma leptin levels in rats with LPS-induced wasting. Thus, ghrelin treatment may provide a new therapeutic approach to the wasting syndrome and critical illness.

Acknowledgments

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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 Junichi 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

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.

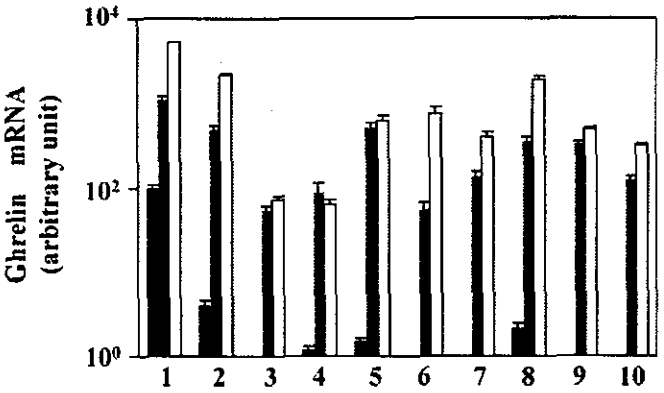

FIG. 1. Preproghrelin mRNA levels in the tissues of control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis. Lanes 1, stomach; 2, small intestine; 3, cerebrum; 4, hypothalamus; 5, pituitary; 6, liver; 7, kidney; 8, lung; 9, heart; and 10, skeletal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

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).

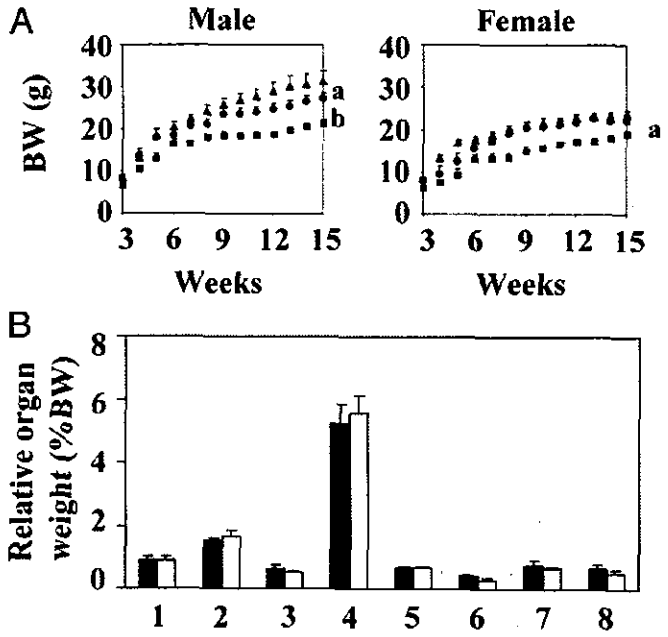

FIG. 2. Body weights (BW) and relative organ weights. A, Body weights of male (*left panel*) and female (*right panel*) control (*triangles*), Tg 9–2 (*circles*), and Tg 10–1 (*squares*) mice (n = 8/group). B, Relative organ weights of 8-wk-old control (*closed bars*) and Tg 10–1 (*open bars*) mice calculated 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*).

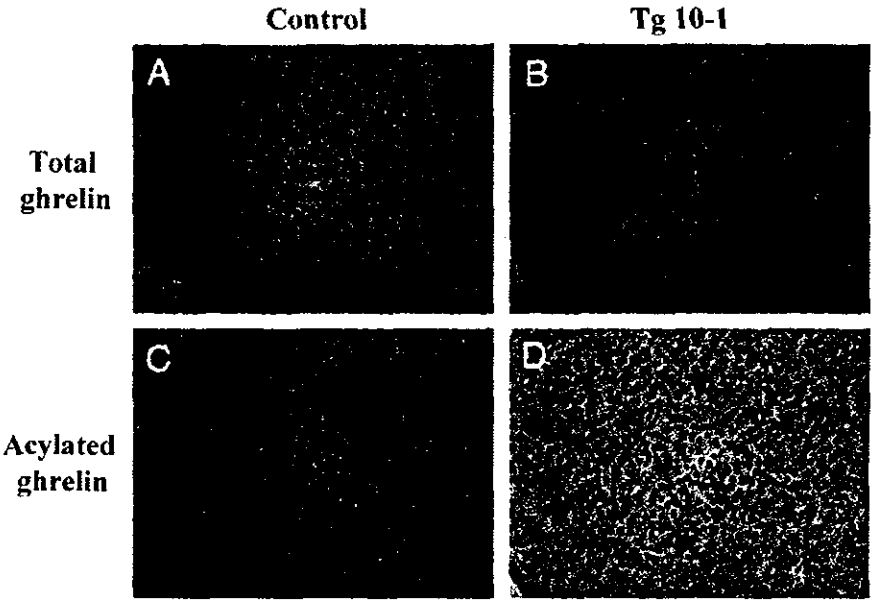

FIG. 3. The localization of total and acylated ghrelin-immunoreactive cells in the pituitary of 8-wk-old male control (A and C) and Tg 10–1 (B and D) mice. A and B, An antiserum raised to ghrelin(13–28) recognizing total (acylated plus des-acyl) ghrelin was used. C and D, An antiserum raised to ghrelin(1–11) 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.

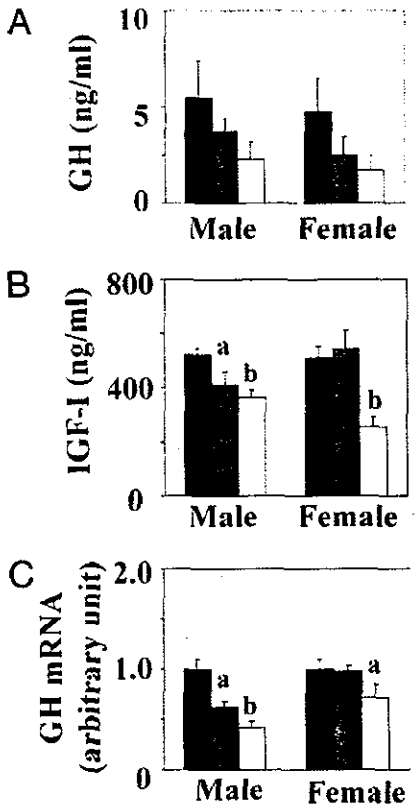

FIG. 4. Serum GH, IGF-I, and pituitary GH mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice (n = 8/group). A, Serum GH levels. B, Serum IGF-I levels. C, Pituitary GH mRNA levels. a, $P < 0.05$; b, $P < 0.01$ (*vs.* control mice).

5. No significant difference was noted in the levels between control and transgenic mice.

Hematoxylin eosin and immunohistochemical staining for GH of the pituitary

Hematoxylin eosin staining is shown in Fig. 5, A and B. The pituitary morphology of Tg 10–1 mice was not different from that of the control mice. Immunohistochemical staining for GH is shown in Fig. 5, C and D. The distribution of GH-immunoreactive cells in the pituitary of Tg mice was similar to that of control mice.

Effects of GHRH and ghrelin on GH release

Control and Tg 10–1 mice were used. Serum GH levels after GHRH administration in male and female Tg 10–1 mice were similar to those of control mice throughout the course of the experiment (Fig. 6A). There was no significant difference in serum GH level at each time point between both male and female Tg-10 and control mice. Serum GH levels 10 min after ghrelin administration in male Tg10–1 and control mice were 63.1 ± 6.8 and 72.6 ± 12.0 ng/ml, respectively (Fig. 6B, *left panel*). The difference was not significant. Serum GH levels 20 min after ghrelin administration in male Tg10–1 and control mice were 30.2 ± 6.7 and 61.2 ± 15.5 ng/ml, respectively, and levels after 30 min were 11.8 ± 1.4 and 21.9 ± 4.1 ng/ml, respectively (Fig. 6B, *left panel*). Both differences were significant ($P < 0.01$). Serum GH levels 10 min after ghrelin administration in female Tg10–1 and control mice were 8.7 ± 3.7 and 52.8 ± 8.2 ng/ml, respectively, and those after 20 min were 29.8 ± 6.3 and 78.5 ± 14.3 ng/ml, respectively (Fig. 6B, *right panel*). Both differences were significant ($P < 0.01$). Serum GH levels 30 min after ghrelin administration in female Tg10–1 and control mice were 22.8 ± 6.3 and 22.3 ± 8.8 ng/ml, respectively (Fig. 6B, *right panel*). The difference was not significant.

TABLE 5. Plasma ACTH, serum TSH, LH, and FSH levels of 8-wk-old control and transgenic mice (n = 8/group)

	Control	Tg 9-2	Tg 10-1
ACTH (pg/ml)	135 ± 35	144 ± 32	123 ± 44
TSH (ng/ml)	3.31 ± 0.06	3.37 ± 0.11	3.49 ± 0.13
LH (ng/ml)	31.5 ± 2.1	30.8 ± 1.7	27.7 ± 2.1
FSH (ng/ml)	268.4 ± 21.8	221.1 ± 43.9	253.5 ± 24.6

Values are given as the mean ± SEM.

Expression of GHS-R in the pituitary

GHS-R mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.56, and 3.46 AU, respectively (Fig. 7). The difference between control and Tg 10–1 mice was significant ($P < 0.01$).

Expression of hypothalamic neuropeptides that regulate GH secretion

GHRH mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 0.88, and, 0.80 AU, respectively (Fig. 8A). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant. Somatostatin mRNA levels of male control, Tg 9–2, and Tg 10–1 mice were 1.00, 1.08, and 0.97 AU, respectively (Fig. 8B). The differences between control and Tg 9–2 mice and control and Tg 10–1 mice were not significant.

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

Male and female C57BL/6 mice were used. Serum GH levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 5.8 ± 1.1 and 7.5 ± 2.0 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 9.2 ± 2.2 and 9.5 ± 1.8 ng/ml, respectively. The difference was not significant either. Serum IGF-I levels after 10 d treatment with saline and des-acyl ghrelin in male mice were 769.3 ± 16.6 and 768.7 ± 21.6 ng/ml, respectively. The difference was not significant. Those with saline and des-acyl ghrelin in female mice were 766.2 ± 13.4 and 719.4 ± 49.1 ng/ml, respectively. The difference was not significant either. Body weights and lengths in des-acyl ghrelin-injected mice were not significantly different from those in saline-injected mice in either males or females (data not shown).

Discussion

We have generated transgenic mouse lines that overexpress preproghrelin mRNA in a wide variety of tissues. The wide tissue distribution of preproghrelin mRNA in trans-

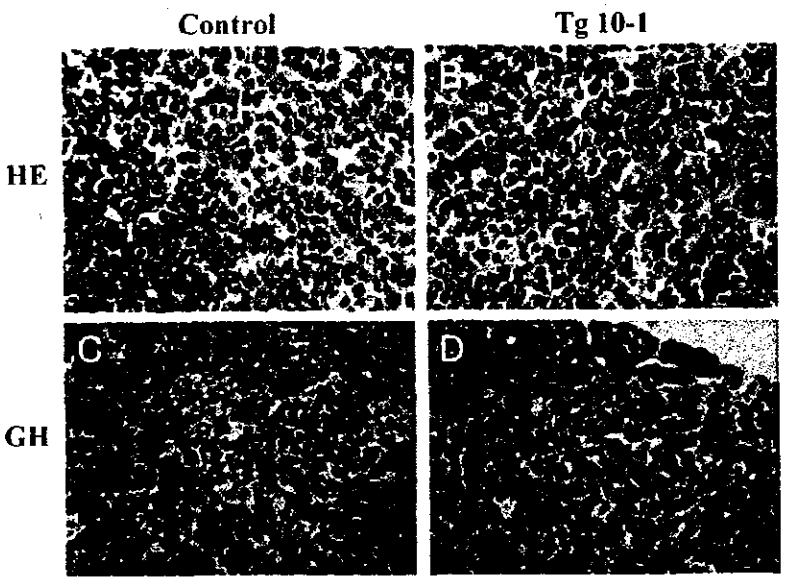

FIG. 5. Morphology of the pituitary and the localization of GH-immunoreactive cells in the pituitary of 8-wk-old male control (A and C) and Tg 10–1 (B and D) mice. A and B, Hematoxylin eosin (HE) staining. C and D, The localization of total and GH-immunoreactive cells in the pituitary. Original magnification, ×40. The immunoreactive cells are stained *brown* by the avidin-biotin complex methods.

genic mice was consistent with previous reports on transgenic mice using the CAG promoter (33, 34). Preproghrelin mRNA expression was increased, especially in Tg 10–1 mice, and its amount in the stomach reached 52-fold of that in control mice. Consistent with the elevated mRNA expression, peptide levels of total ghrelin (des-acyl plus acylated ghrelin) in various tissues were also elevated in transgenic mice. Plasma total ghrelin levels in transgenic mice showed marked results. Those in transgenic mice showed 10- and 44-fold of those in control mice. We originally intended to generate mice overexpressing biologically active ghrelin. Unexpectedly, acylated ghrelin levels were not changed in all tissues examined and plasma of transgenic mice, compared with those of control mice, indicating that transgenic mice overexpress only des-acyl ghrelin. The expression of acylated ghrelin has been reported in a small number of tissues, such as the stomach (X/A cells), duodenum, hypothalamus, and pancreatic α-cells (1, 31, 39, 40). These reports and our present data suggest that only a limited number of cell lineages may able to process proghrelin or acylate ghrelin. The underlying mechanism by which ghrelin is acylated is unknown to date. Further study is needed to clarify the mechanism of the acylation.

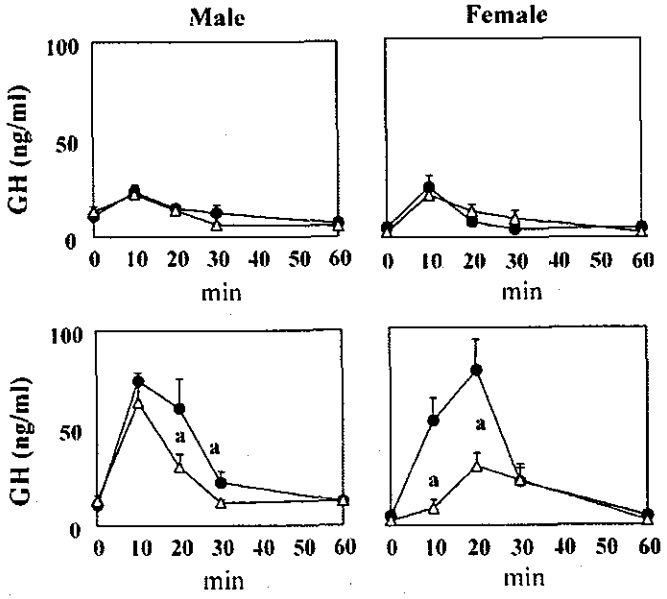

FIG. 6. The responses of GH to GHRH and ghrelin in 8-wk-old control (*closed circles*) and Tg 10–1 (*open triangles*) mice. A, Time course of serum GH levels after iv injection of 60 μg/kg GHRH (n = 8/each point). B, Time course of serum GH levels after iv injection of 40 μg/kg ghrelin (n = 8/each point). a, $P < 0.01$ (*vs.* control mice).

The acylation of ghrelin is assumed to be essential for its actions, and des-acyl ghrelin, which lacks the modification, is devoid of endocrine actions, based on previous studies (1, 41). However, recent studies indicated that des-acyl ghrelin may have some actions. Des-acyl ghrelin as well as acylated ghrelin causes a significant inhibition of cell proliferation in human breast carcinoma cell lines (29) and inhibits cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKT (30). In addition, one study (42) reported that acylated and des-acyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of known GHS-Rs. Moreover, another study (28) indicated that des-acyl ghrelin may offset the action of acylated ghrelin on insulin secretion. Ghrelin has been shown to induce a reduction in serum insulin levels. In the study, coadministration of acylated plus des-acyl ghrelin did not re-

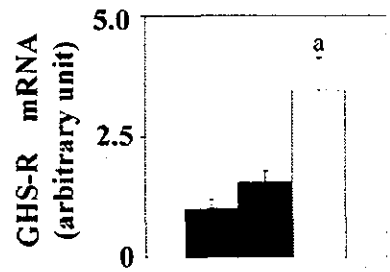

FIG. 7. Pituitary GHS-R mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis (n = 8/group). a, $P < 0.01$ (*vs.* control mice).

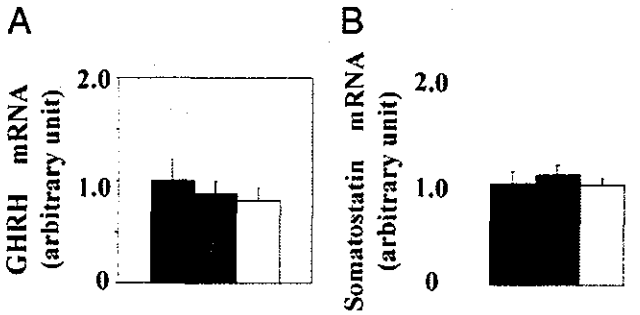

FIG. 8. Hypothalamic GHRH and somatostatin mRNA levels in 8-wk-old control (*closed bars*), Tg 9–2 (*shaded bars*), and Tg 10–1 (*open bars*) mice quantified by real-time PCR analysis. A, GHRH mRNA levels (n = 8/group). B, Somatostatin mRNA levels (n = 8/group).

sult in any changes in serum insulin levels in humans, suggesting that ghrelin action on insulin is modulated by des-acyl ghrelin.

The present study indicates that transgenic mice overexpressing des-acyl ghrelin show small phenotype. Longitudinal growth was the most reduced in female Tg 10–1 mice (20% reduction from control mice). The phenotype was not associated with changes in BMIs. These mice did not show decreased food intake or decreased body fat mass. In addition, they showed normal nutritional condition, based on their biochemical parameters, including blood glucose, serum total protein, and total cholesterol levels. These data indicate that the small phenotype of transgenic mice is not attributed to poor nutritional condition.

Serum IGF-I levels were significantly reduced in male and female transgenic mice, compared with control mice. Female Tg 10–1 mice had no less than 50% reduction in serum IGF-I levels, compared with control mice. Although the differences in serum GH levels between control and transgenic mice were not statistically significant, probably because of the pulsatile character of GH secretion, the levels tended to be reduced in transgenic mice, compared with control mice, and the mean GH level of Tg10–1 mice was only 50% of that of control mice. It should be emphasized that Tg 10–1 mice showed lower serum GH levels than Tg 9–2 mice. Body weights and lengths of the former were more reduced than the latter. It should be also noted that the former showed higher des-acyl ghrelin expression than the latter. Reduced pituitary GH mRNA levels in transgenic mice support the observation. The GH-IGF-I axis-specific alteration in transgenic mice was also indicated by the measurement of other anterior pituitary hormones than GH. Plasma ACTH, serum TSH, LH, and FSH levels were not altered.

The size and morphology of the pituitary including the somatotrope populations of transgenic mice were similar to those of control mice. These data indicated that there is no apparent change, suggesting developmental problems in the pituitary of transgenic mice.

Responses of GH to GHRH and ghrelin in transgenic mice exhibited intriguing results. Transgenic mice showed normal response of GH to GHRH. Alternatively, if we consider that the basal GH levels are lower in transgenic mice, the similar maximal response might indicate that they are hyperresponsive to GHRH. It is not likely that an insufficient dose of GHRH induced submaximal response of GH in both control and transgenic mice, judging from previous reports (43). On the other hand, the responses of GH to ghrelin were reduced in transgenic mice. It is noteworthy that the reduction was much greater in female transgenic mice than in male mice, if we take their serum IGF-I levels into account. Taken together our results and these reports indicate that overexpression of des-acyl ghrelin in our mice may result in reduction of GH response to endogenous ghrelin, and it may result in the reduced serum IGF-I levels in transgenic mice.

The reduced GH response to ghrelin in transgenic mice could be due to down-regulated the GHS-R. However, the pituitary GHS-R mRNA levels in the transgenic mice were rather elevated. It is not likely that overexpressed des-acyl ghrelin acts as a blocking agent to the GHS-R because ^{125}I-labeled acylated ghrelin bound to the GHS-R cannot be displaced by des-acyl ghrelin (20). Overexpressed des-acyl ghrelin may have some effects on endogenous GH secretion, modifying the action of endogenous ghrelin in transgenic mice via, for instance, another receptor or modulation of the signal transduction pathway after the GHS-R.

Previous reports indicated that the hypothalamus plays a critical role in the stimulatory effect of ghrelin on GH secretion as well as the pituitary (21, 22, 23). Because GH secretion is regulated chiefly by two hypothalamic hormones, GHRH and somatostatin, the expression of these hormones could be altered in transgenic mice. We could not find any significant difference in either GHRH or somatostatin mRNA levels between control and transgenic mice. These data might suggest that overexpressed des-acyl ghrelin acts on not only the pituitary but also the hypothalamus in the transgenic mice, judging from the fact that hypothalamus GHRH mRNA were not elevated, and somatostatin mRNA levels were not decreased despite the decreased serum GH levels.

We could not show, unfortunately, that continuous ip infusion of des-acyl ghrelin has some effect on serum GH and IGF-I levels or body weights. It should be noted, however, that plasma des-acyl ghrelin levels in transgenic mice reached 10- and 50-fold of those in control mice. Administration of a higher dose of des-acyl ghrelin, or longer administration, might result in alteration in the GH-IGF-I axis. On the other hand, the phenotype of transgenic mice might reflect direct effects of ubiquitous expression of des-acyl ghrelin. It should also be noted that high levels of des-acyl ghrelin were detected in a various tissues, especially in the pituitary, as well as in plasma of transgenic mice. The des-acyl ghrelin immunoreactive pituitary cells might play an important role in the mechanism for the altered GH-IGF-I axis in a paracrine or autocrine manner. It should be pointed out that preproghrelin mRNA is reported to be expressed in the normal pituitary (44), as we showed in the present study, suggesting its physiological role in GH secretion. The phenotype of transgenic mice may reflect the role. Further study is needed for this issue.

The mechanism underlying the sexual dimorphism in the responses of GH to ghrelin in transgenic mice is not fully understood. It might be due to the gender difference in the secretory regulation of GH. Female mice have been reported to be different from male mice in that they have noncyclical

and rather low somatostatin output and that GHRH plays a dominant role in it (45). There might be a GHRH-dependent mechanism for the reduced response in transgenic mice. Indeed, one recent report (26) indicated that transgenic rats expressing an antisense GHS-R mRNA in the hypothalamic arcuate nucleus show marked gender difference in GH secretion. Although there was no significant difference in pulse frequency and baseline levels of GH between male control and transgenic rats, female transgenic rats showed lower baseline levels and fewer pulses of GH than female control rats (26).

The 94-amino acid proghrelin is cleaved to yield ghrelin. One previous study (46) demonstrated that C-terminal proghrelin peptides are present in the human circulation. Transgenic mice in the present study would also overexpress these peptides. We have not excluded the possibility that the phenotype of transgenic mice might be due to the effects of these peptides.

In conclusion, the present study demonstrates that transgenic mice overexpressing des-acyl ghrelin show small phenotype and altered GH-IGF-I axis. These observations may indicate a role of des-acyl ghrelin in the regulation of GH secretion.

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Cloning and characterization of the 5′-flanking region of the human ghrelin gene

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Abstract

Ghrelin, a novel growth hormone releasing peptide, was recently isolated from stomach. We have cloned and characterized the 5′-flanking region, containing from −2000 to −1 upstream from the translation start site of the human ghrelin gene. There was neither typical GC nor CAAT box but there were a TATATAA element and putative binding sites for several transcription factors. Ghrelin promoter was activated only in human stomach derived ECC10 cells among several cell lines examined. Functional analysis showed that promoter activity was increased by deletion of nucleotides from −2000 to −605 whereas it was decreased by further deletion and that the TATATAA element is not functioning. Glucagon and its second messenger cAMP enhanced the promoter activity, suggesting that stimulated transcription of ghrelin gene by glucagon might be responsible for increased ghrelin production during fasting at least in part. These initial characterizations will facilitate further studies of the regulatory mechanisms for ghrelin gene expression.

Keywords: Ghrelin; Growth hormone; Stomach; Promoter; Transcription; Glucagon

Ghrelin is an acylated 28-amino acid peptide recently identified in human and rat stomachs as an endogenous ligand for the GH secretagogue receptor [1]. Ghrelin increases secretion of GH, food intake, and controls energy balance [1–3]. It has been reported that ghrelin produced in the X/A cells of the stomach is a major source of plasma ghrelin [4,5]. Other than stomach, ghrelin is expressed in the pituitary gland, arcuate nucleus of the hypothalamus, pancreas, kidney, and placenta [1,5–8]. Also, some tumors produce ghrelin [9,10]. Papotti et al. [10] reported that RT-PCR showed ghrelin mRNA in all gastrointestinal carcinoids examined.

It is well known that plasma ghrelin levels were increased during fasting and decreased by refeeding. However, factors which regulate the production of ghrelin have not been clarified. While there is a report to show that ghrelin expression in the stomach is up-regulated by insulin and leptin [11], it is also reported that ghrelin expression in the stomach was down-regulated by insulin [12] and leptin [13]. Therefore, it seems that insulin and leptin are not the main regulators for ghrelin production.

To understand the transcriptional regulation of the human ghrelin gene expression, we have cloned and characterized the 5′-flanking region of the human ghrelin gene.

Materials and methods

Materials and cell culture. All chemicals were obtained from Sigma. Fetal calf serum (FCS), horse serum, RPMI1640, Dulbecco's modified Eagle's medium (DMEM), and Ham's F-10 were obtained from Life Technologies (Tokyo, Japan). The human gastric carcinoid derived cell line ECC10 cells, which were obtained from Riken Cell Bank (Cell No. RCB0983), human gastric adenocarcinoma derived MKN1, and MKN 45 cells were maintained in RPMI1640 with 10% calf serum. Rat pituitary derived GH3 cells were maintained in Ham's F-10 medium supplemented with 15% horse serum and 2.5% FCS. COS7, CHO,

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PC3, F9, HeLa, and JEG3 cells, which were derived from monkey kidney, Chinese hamster ovary, human prostate gland, mouse testis, human cervix, and human trophoblast, respectively, were maintained in DMEM with 10% calf serum.

Cloning of the 5′-flanking region of the human ghrelin gene. We have cloned the 5′-flanking region of the human ghrelin gene with polymerase chain reaction (PCR)-based gene walking method (Human Genome Walker kit; Clontech, Palo Alto, CA). The 5′-flanking region of the human ghrelin gene was amplified from the five Human Genome Walker genomic libraries with two consecutive rounds of PCR using the adaptor primers AP1 and AP2 and the gene-specific reverse primers GSP1 (5′-CAGGAGGCTGCAGACGGTCCC-3′, +32 to +13) and GSP2 (5′-TGGGGAGGGCATGGCCTCAGC-3′, +12 to −9) (the translation start site was set at +1) (see Fig. 1A). The gene-specific oligonucleotide primers were synthesized based on sequences of the human ghrelin cDNA (GenBank Accession No. AB029434). The PCR product was subcloned into the pT7 blue vector (Novagen, Madison, WI) and sequenced with a DNA autosequencer (ABI prism377A; Perkin–Elmer). Because the sequence of the PCR product was found on a *Homo sapiens* chromosome 3 clone RP11-1082A18 (GenBank Accession No. AC090841), we amplified the 5′-flanking region using GSP3 (5′-GTTTTTCTTCCAGCGAAATAAAG -3′, −2000 to −1978 according to the draft sequence) and GSP4 (5′-GGCCTCA GCTGGGTTGCAGAC-3′, −1 to −21). Human Genome Walker genomic library (No. 1) was used as a template. The PCR product was subcloned into the pT7 blue vector and sequenced.

Rapid amplification cDNA ends. The 5′-end of the human ghrelin cDNA was determined with the rapid amplification of cDNA end (5′-RACE) method (see Fig. 3A). Human stomach Marathon Ready cDNA (Clontech) was amplified with PCR using the adaptor primer AP1′ and cDNA specific primer GSP5 (5′-GGCTCAGGAAGCTGG AGCCTG-3′). The reaction involved 1 min of denaturation at 94 °C, followed by 5 cycles consisting of 30 s of denaturation at 94 °C and 4 min of annealing and extension at 72 °C, 5 cycles consisting of 30 s of denaturation at 94 °C and 4 min of annealing and extension at 70 °C, and 25 cycles consisting of 20 s of denaturation at 94 °C and 4 min of annealing and extension at 68 °C. The PCR products were diluted to one-fiftieth and then subjected to the secondary PCR with the nested primers AP2′ and GSP6 (5′-CCATGGCCAAGTCCAGCCAGA-3′)

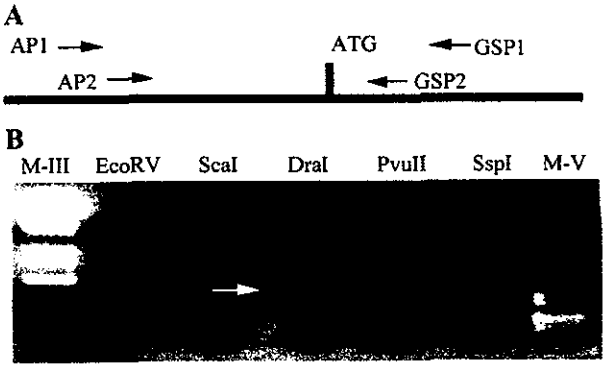

Fig. 1. Cloning of the 5′-flanking region of human ghrelin gene. (A) Schematic diagram representing the positions of primers used for the PCR-based gene walking method. The 5′-flanking region of the human ghrelin gene was amplified from the five Human Genome Walker genomic libraries with two consecutive rounds of PCR using the adaptor primers and the gene-specific reverse primers. (B) Results of agarose gel (1%) electrophoresis and ethidium bromide staining of amplification product are shown. We obtained a PCR product extending to the first *Dra*I site (arrow) containing from −605 to −1 of the human ghrelin gene (the translation start site was set at +1). DNA molecular size markers III and V were run in the left and the right lanes, respectively.

using the same protocol. The PCR products were sequenced after being subcloned into the pT7 blue vector.

Reporter gene construction and transient expression assays. A fragment of the 5′-flanking region of the human ghrelin gene (−2000 to −1) was subcloned into a reporter plasmid, pGL3-Basic vector (Promega, Tokyo, Japan), to be fused to the luciferase gene (−2000-Ghrelin-Luc). Deletion mutant plasmids were generated by PCR (−1000, −605, −301, −200, and −150-Ghrelin-Luc). Two mutant forms of −605-Ghrelin-Luc, which have mutated TATATAA element (−585 to −579), were made as described previously [14] using −605-Ghrelin-Luc as a template. Mutant sense primer 1 (5′-GCCAGTCATCCTGTCTGAGG ACCTGACAG-3′) and mutant anti-sense primer 1 (5′-CTGTCAGGT CCTCAGACAGGATGACTGGC-3′), or mutant sense primer 2 (5′-GCCAGTCATCCGACATGGGGACCTGACAG-3′) and mutant anti-sense primer 2 (5′-CTGTCAGGTCCCCATGTCGGATGACT GGC-3′) were used. The correct sequence of these mutant plasmids was confirmed by DNA sequencing. After transfection with Lipofectamine Reagent (Invitrogen, Tokyo, Japan), the cells were grown in a normal growth medium, or the medium containing 0.1% BSA instead of serum with vehicle or 10 nM glucagon, or the medium with vehicle or 1 mM CPT-cAMP, or the four medium containing different concentrations of glucose (50, 100, 200, and 400 mg/dl) for 24 h. Luciferase activity was determined in a Turner design luminometer TD-20/20 (Promega) using the dual luciferase assay system (Promega) and normalized with the luciferase activity of co-transfected pRL-CMV containing the cDNA encoding *Renilla* luciferase (Promega). In each transfection, 2 μg of the reporter plasmids and 20 ng pRL-CMV were co-transfected into ECC10, COS7, CHO, PC3, F9, HeLa, JEG3, or GH3 cells cultured in the 35-mm dishes.

Reverse transcription and amplification of cDNA. Total RNA was prepared from cultured ECC10 using Trizol according to the supplier (Life Technologies). The first strand cDNA was synthesized from 4 μg total RNA using random hexamers (GeneAmp RNA PCR Core Kit, Perkin–Elmer, Foster City, CA). PCR was carried out in a 50 μl reaction mixture containing 4 μl of the above first-strand cDNA, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP mix, 4 pmol of each primer, and 2.5 U *Taq* DNA polymerase (Gibco). The sequences of primers for the amplification of the human glucagon receptor cDNA were 5′-TGG ATGGCGAGGAGATTGAG-3′ and 5′-GCGGACGAAGATGAAG AAGT-3′ [15]. The sequences of primers for the amplification of the human insulin receptor cDNA were 5′-ATGAACTCCTTCAATTAT AC-3′, and 5′-TGGTGGAAAGTACTCCTCCCG-3′ [16]. The sequences of primers for the amplification of the human leptin receptor cDNA were 5′-CAGAAGCCAGAAACGTTTGAG-3′ and 5′-AGC CCTTGTTCTTCACCAGT-3′ [17]. The sequences of primers for the amplification of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were 5′-CCCTTCATTGACCTCAACTA-3′ and 5′-GCCAGTGAGCTTCCCGTTCA-3′ [18]. The PCR involved 30 cycles consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 58 °C, and 1 min of extension at 72 °C. Autoclaved water and Human liver Marathon Ready cDNA (Clontech) were used as a negative and a positive template, respectively. These PCR products were verified with DNA sequencing.

Statistical analysis. The data were expressed as means ± SE. Statistics were analyzed by one-way repeated measures analysis of variance with a significance level of 0.05.

Results

Cloning of the 5′-flanking region of the human ghrelin gene

We obtained a PCR product extending to the first *Dra*I site which contained −605 to −1 upstream from the

translation initiation site of the human ghrelin gene (Fig. 1B). Another PCR product which contained −2000 to −1 upstream from the translation initiation site of the human ghrelin gene was successfully amplified by GSP3 and GSP2. DNA sequences of the latter PCR product completely matched not only with the sequence, which are identified on a *Homo sapiens* chromosome 3 clone RP11-1082A18 (GenBank Accession No. AC090841),

Fig. 2. The nucleotide sequences of the 5′-flanking region of the human ghrelin gene. The translation start site was set at +1. The major 5′-end of the 5′-RACE products is denoted by an asterisk. The putative binding sites for several transcription factors were found.

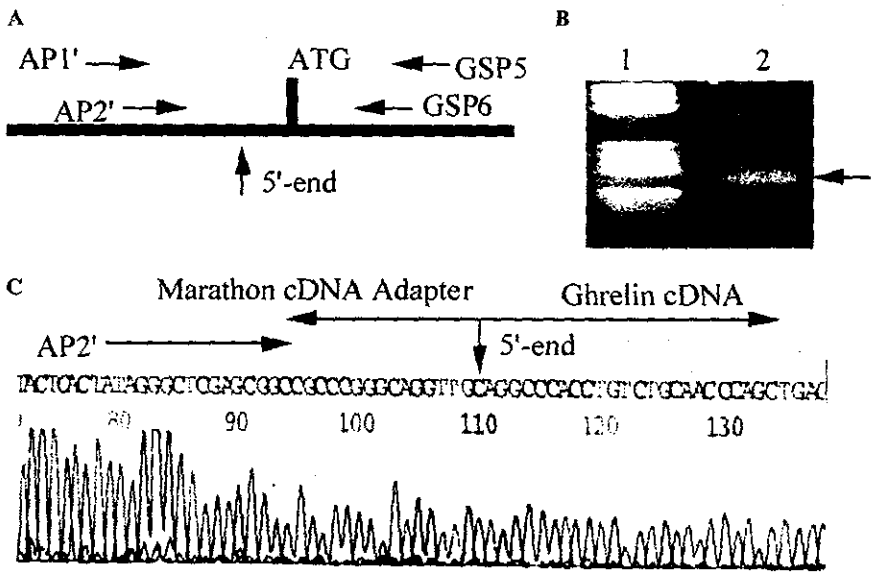

Fig. 3. 5′-RACE of the human ghrelin. (A) Schematic diagram representing the positions of primers used for 5′-RACE. (B) Human stomach Marathon cDNA was subjected to two sets of PCR. Results of agarose gel (1%) electrophoresis and ethidium bromide staining of amplification products are shown in lane 2. DNA molecular size marker V was run in lane 2. (C) Amplified products were subcloned and sequenced, and representative data are shown.

but also with DNA sequences of the former PCR product which contained −605 to −1 upstream from the translation initiation site of the human ghrelin gene (Fig. 2).

The 5′-end of cDNA of the human ghrelin gene was determined by the 5′-RACE. The human stomach cDNA was amplified by the second PCR using AP2′ and GSP5 and the PCR products were subcloned into the pT7 blue vector. All of the eight clones sequenced showed that the 5′-end of cDNA of the human ghrelin gene was −32 from the translation start site, suggesting that −32 seemed to be a major transcription start site (Fig. 3).

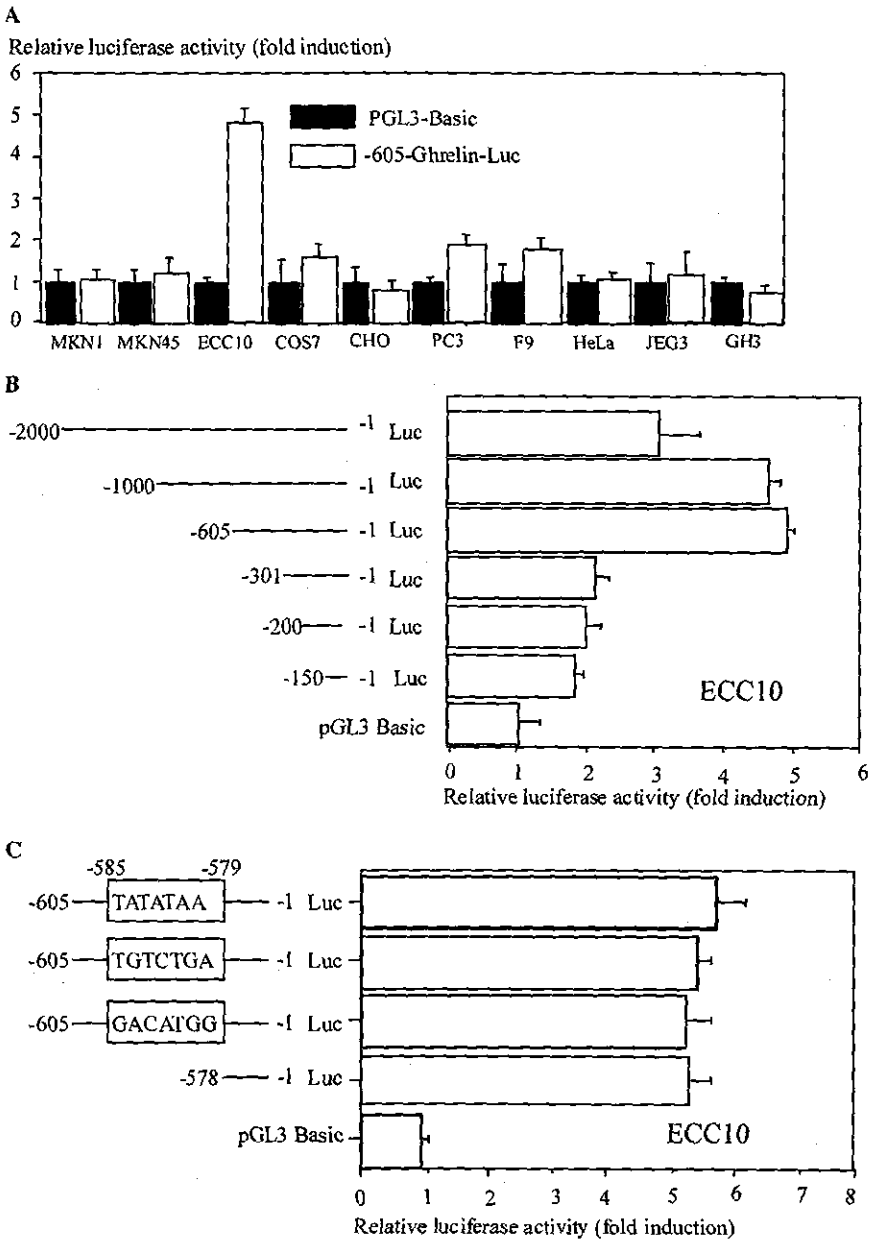

Fig. 4. Functional analysis of the human ghrelin promoter. (A) Cell specificity of the human ghrelin promoter activity. Plasmids PGL3-Basic containing −605 to −1 of the human ghrelin gene (−605-Ghrelin-Luc) were transiently transfected into several cell lines. (B) Deletion analysis of the human ghrelin promoter using ECC10. The schematic diagram on the left represents each deletion construct of the human ghrelin gene fused into the upstream region of the luciferase gene. (C) TATATAA element is not important for promoter activity of the human ghrelin gene. There is a TATA box like element (TATATAA) from −585 to 579. To clarify its role for the human ghrelin gene transcription, TATATAA was mutated to TGTCTGA or GACATGG, or deleted. (A–C) In each transfection, 2 μg of the reporter plasmids was transfected into ECC10, COS7, CHO, PC3, F9, HeLa, JEG3, or GH3 cells cultured in the 35-mm dishes. Twenty ng of pRL-CMV containing the cDNA encoding *Renilla* luciferase was co-transfected to normalize the luciferase activity in each transfection. Experiments were performed in triplicate and values (means ± SE) are expressed as multiples of induction relative to luciferase activity when promoterless PGL3-Basic was transfected.