male GP-Tag Tg mice at 12 wk of age (Fig. 3*E*). Plasma ghrelin levels of 12-wk-old male GP-Tag Tg mice correlated to body weight ($r = 0.574$, $P < 0.05$, $n = 13$; Fig. 3*F*). The regression coefficient of the regression line of GP-Tag Tg mice was bigger than that of nontransgenic littermates ($t = 2.08$, $P < 0.05$) These results indicate that regulation of plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were preserved, at least with regard to feeding status, body weight, and sex difference.

Body weights, body composition, and food intake of GP-Tag Tg mice. There was no difference in body weights between male GP-Tag Tg mice and controls until 12 wk of age ($n = 22$–34; Fig. 4*A*). After 13 wk of age, the body weights of the male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates concomitantly with the decrease in the food intakes of male GP-Tag Tg mice after 11 wk of age (Fig. 4, *A* and *B*). When the body compositions were examined by computed tomography scan, fat masses were significantly reduced in 15-wk-old male GP-Tag Tg mice ($P < 0.05$, $n = 7$–9; Fig. 4*C*), whereas lean body masses and body lengths were not changed (NS, $n = 7$–9; Fig. 4, *D* and *E*). We also examined hypothalamic mRNA levels of neuropeptide Y (NPY), agouti-related protein (AgRP), and GHS-R in 12-wk-old male GP-Tag Tg mice. No significant changes were observed in these mRNA levels (NS, $n = 7$; Fig. 4*F*). When 15-wk-old male GP-Tag Tg mice were injected with ghrelin, the food intake was stimulated to the same extent as in controls (NS, $n = 10$–18; Fig. 4*G*). Plasma leptin levels of 15-wk-old male GP-Tag Tg mice were significantly lower than controls ($P < 0.05$, $n = 6$; Fig. 4*H*).

GH-IGF-I axis in GP-Tag Tg mice. Serum IGF-I levels of 12- and 15-wk-old male GP-Tag Tg mice were significantly higher than those of nontransgenic littermates ($P < 0.05$, $n = 7$–8, and $P < 0.05$, $n = 6$–7, respectively; Fig. 5*A*). Although basal serum GH levels of 15-wk-old male GP-Tag Tg mice were not significantly different from controls, serum GH levels after GHRH injection tended to be high ($P = 0.077$, $n = 8$–13), which was not observed after ghrelin injection (Fig. 5*B*). We then investigated the effects of chronic ghrelin elevation on hypothalamic and pituitary mRNA levels of components involved in GH regulation. There were no differences in hypothalamic mRNA levels of GHRH and somatostatin or in pituitary mRNA levels of GH and GHRH receptor (GHRH-R) between 15-wk-old male GP-Tag Tg mice and their littermates

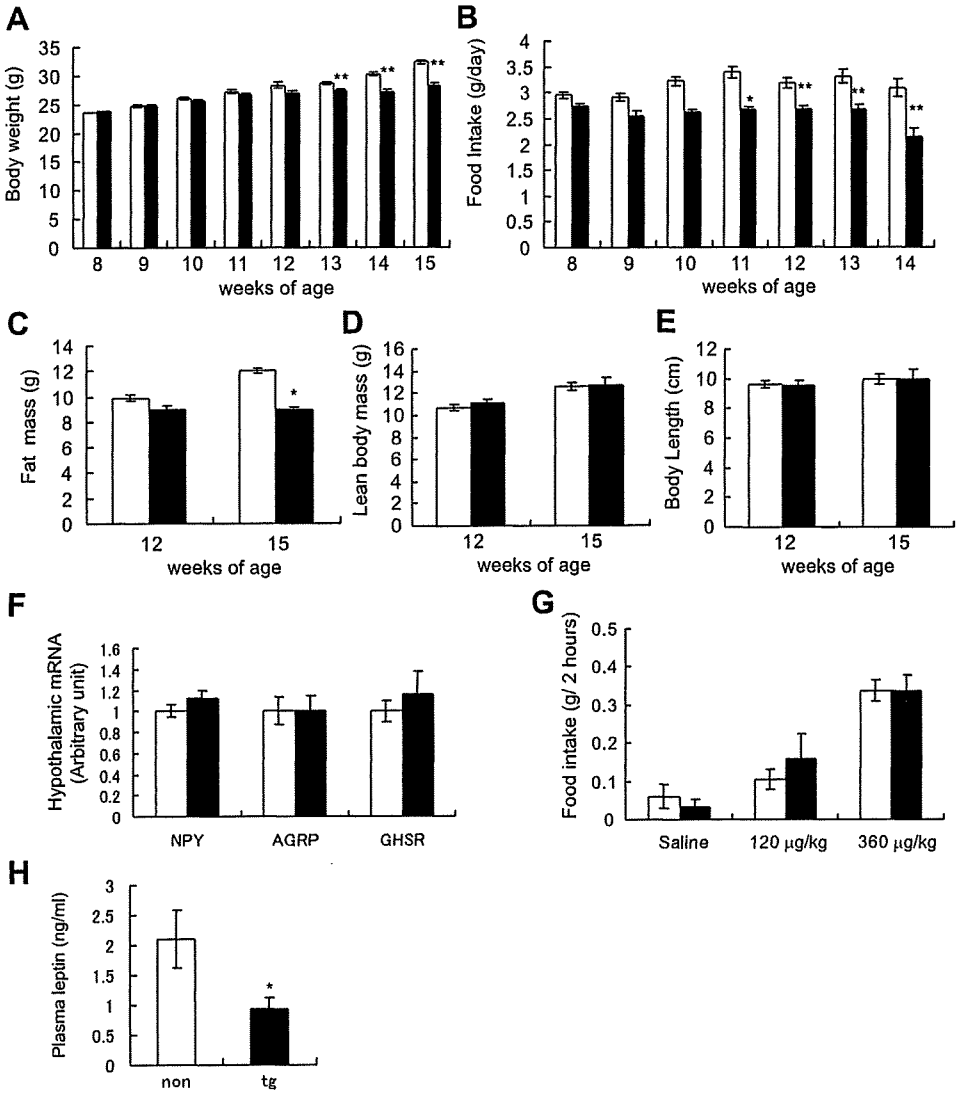

Fig. 4. Body weights, body compositions, and food intakes of GP-Tag Tg mice. *A*: body weights of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 22$–34. *B*: daily food intakes of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 19$–26. *C* and *D*: fat mass (*C*) and lean body mass (*D*) determined by animal computed tomography scan of 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars). *E*: body length of 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7$–9. *F*: hypothalamic mRNA levels of neuropeptide Y (NPY), agouti-related protein (AgRP), and growth hormone secretagogue receptor (GHS-R) in 12-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7$. *G*: food intake for 2 h after injection of ghrelin (120 or 360 μg/kg or saline; $n = 10$–18). *H*: plasma leptin levels in 15-wk-old male Tg mice (black bars) and non littermates (open bars); $n = 6$–7. $^*P < 0.05$, $^{**}P < 0.01$ compared with nontransgenic littermates.

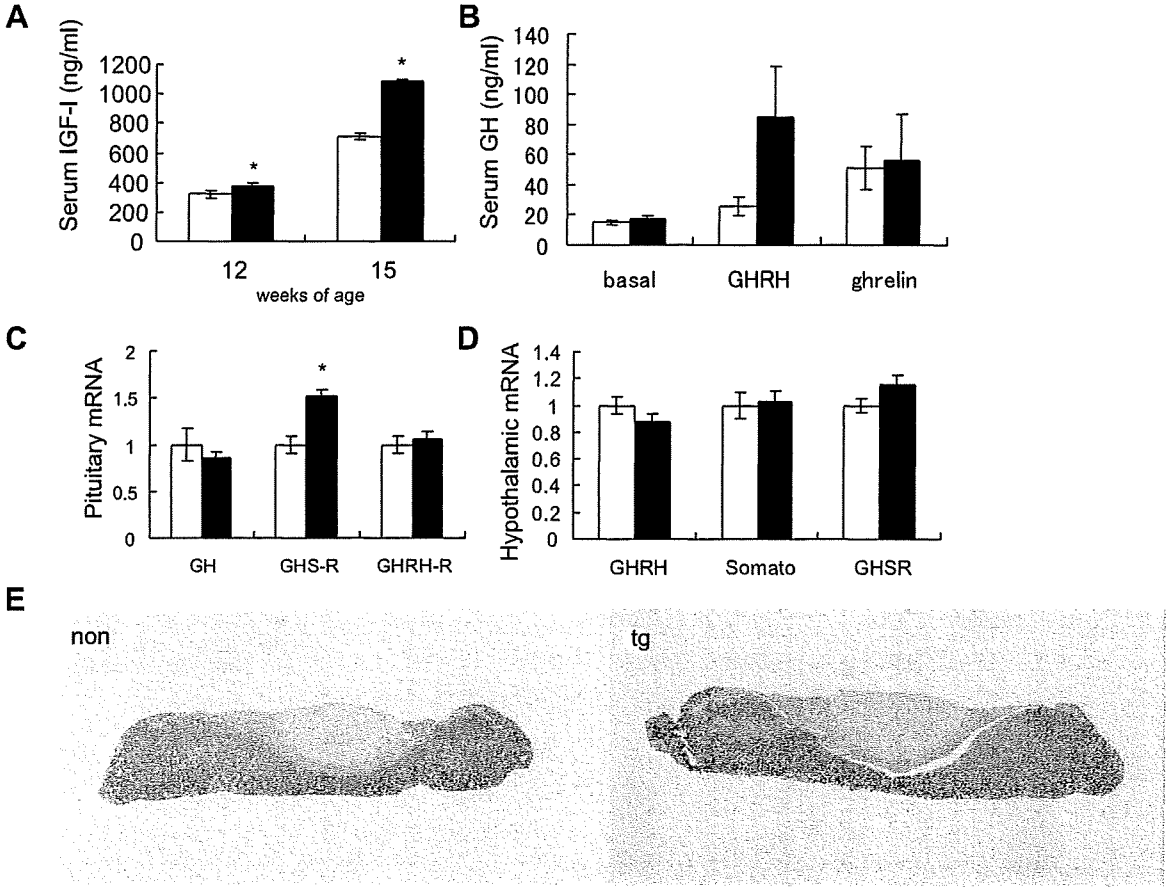

Fig. 5. GH-IGF-I axis in GP-Tag Tg mice. *A*: serum IGF-I levels in male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7–8$. *B*: serum GH levels at basal state and at 15 min after subcutaneous injection of GH-releasing hormone (GHRH) or ghrelin in male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 8–13$. *C*: pituitary mRNA levels of GH, GHS-R, and GHRH-R in 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7$. *D*: hypothalamic mRNA levels of GHRH, somatostatin (somato), and GHS-R in 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7$. *E*: pituitary sections of 15-wk-old male Tg mice and non littermates immunostained with anti-GH antibody. $^*P < 0.05$ compared with non littermates.

(NS, $n = 7$; Fig. 5, *C* and *D*). Although plasma ghrelin level was elevated, pituitary GHS-R mRNA level was upregulated in GP-Tag Tg mice ($P < 0.05$, $n = 7$; Fig. 5*C*). We also examined pituitaries of 15-wk-old male GP-Tag Tg mice by immunohistochemical analysis. There were no obvious differences in somatotroph cell number or staining intensity of GH between GP-Tag Tg mice and nontransgenic littermates (Fig. 5*E*).

Glucose metabolism in GP-Tag Tg mice. Blood glucose levels of 15-wk-old male GP-Tag Tg mice were significantly higher than controls ($P < 0.05$, $n = 10$; Fig. 6*A*), although those of 9-wk-old male GP-Tag Tg mice were comparable with the controls (non-Tg vs. Tg: 96.0 ± 4.7 vs. 100.6 ± 4.7, $P = 0.51$, $n = 9$). Intraperitoneal glucose tolerance tests showed significantly higher blood glucose levels in 15-wk-old male GP-Tag Tg mice ($P < 0.05$, $n = 6–11$; Fig. 6*B*). To estimate the insulin sensitivity of GP-Tag Tg mice, we performed an insulin tolerance test. The blood glucose levels after insulin injection in 15-wk-old male GP-Tag Tg mice were suppressed to the same level of those in controls (NS, $n = 5–8$; Fig. 6*C*). Although basal insulin levels of 15-wk-old male GP-Tag Tg mice were not significantly different from those of control mice, those after glucose injection were significantly suppressed in GP-Tag Tg mice ($P < 0.05$, $n = 7–8$; Fig. 6*D*). Pancreatic mRNA and protein levels of insulin in GP-Tag Tg were comparable with those of nontransgenic littermates (NS, $n = 6–8$; Fig. 6, *E* and *F*).

DISCUSSION

In this study, we successfully established a mouse model of ghrelinoma, GP-Tag Tg mouse. GP-Tag Tg mice exhibited chronic elevation of circulating ghrelin with physiological regulation. The elevation of circulating ghrelin in GP-Tag Tg mice (~10-fold elevation) was much higher than that in bacterial artificial chromosome transgenic mice created by Bewick et al. (5) (only ~1.5-fold elevation). Nevertheless, the levels of circulating ghrelin in GP-Tag Tg mice can be considered to be within the physiological range since the highest level of plasma ghrelin observed in the anorexia patients is about seven times higher than those of normal controls (3). One may be confused by low ghrelin mRNA levels and low ghrelin production per milligram of tissue in the stomachs of GP-Tag Tg mice. In general, when the cell cycle progresses, endocrine cell produces far less amounts of hormone since the hormone production occurs mainly at the G_0/G_1 phase of the cell cycle. Since the hyperproliferaing ghrelin-producing cells in GP-Tag Tg mice were forced to proliferate by SV40 T-antigen, which suppresses RB protein and p53, promoting cell cycle progres-

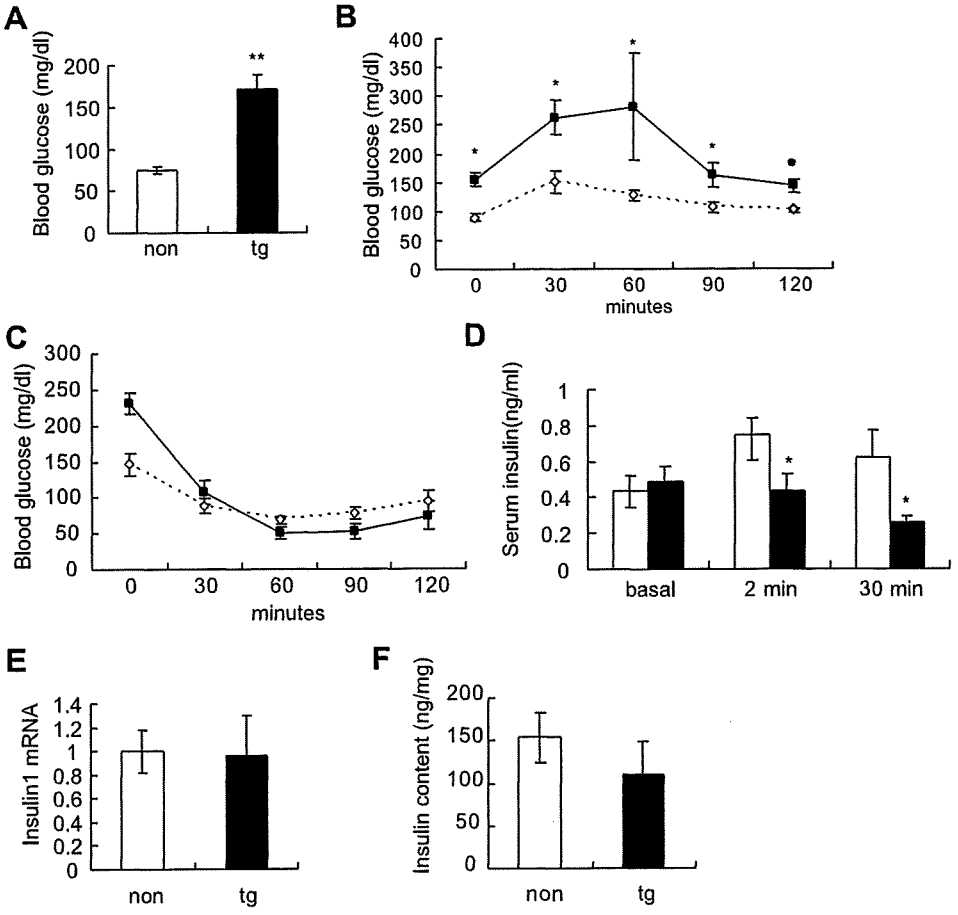

Fig. 6. Glucose metabolism in GP-Tag Tg mice. *A*: fasting blood glucose levels in 15-wk-old male Tg (black bar) and in non (open bar); $n = 7–10$. *B*: glucose tolerance tests in 15-wk-old male GP-Tag Tg mice (■) and in their nontransgenic littermates (◇); $n = 6–11$. *C*: insulin tolerance tests in male GP-Tag Tg mice (■) and in their nontransgenic littermates (◇); $n = 5–8$. *D*: serum insulin levels at basal, at 2 min, and at 30 min after intraperitoneal glucose injection in 15-wk-old male GP-Tag Tg mice (black bars) and in their nontransgenic littermates (open bars); $n = 7–8$. *E* and *F*: the mRNA (*E*) and the protein levels (*F*) of insulin in the pancreata of 15-wk-old male Tg mice (black bars) and in their non littermates (open bars); $n = 6–8$. $^*P < 0.05$, $^{**}P < 0.01$ compared with nontransgenic littermates.

sion, the amount of ghrelin production per cell was low. However, since the cell number was extremely increased, the net product by stomach was eventually elevated.

Several lines of evidence suggest that the GH-IGF-I axis is suppressed in the decreased GHS-R signaling state (28, 32). It has not yet been clear, however, whether chronic elevation of ghrelin within the physiological range could stimulate the GH-IGF-I axis. In this study, we found that adult GP-Tag Tg mice with elevated circulating ghrelin level showed elevated serum IGF-I level. Serum IGF-I level is regulated not only by GH but also by nutritional status. Malnutrition suppresses serum IGF-I level, whereas overnutrition elevates it (16). Since the nutritional state of GP-Tag Tg mice was poor because of decreased food intake, the elevated serum IGF-I levels in adult GP-Tag Tg mice are considered not to be due to overnutrition but to be due to activation of GH-IGF-I axis. Our findings indicate that chronic elevation of circulating ghrelin within the physiological range can activate the GH-IGF-I axis. As far as we know, this is the first report demonstrating that increased levels of circulating ghrelin within the physiological range can elevate serum IGF-I levels in rodent.

The GH-releasing action of ghrelin requires GHRH (11), and when coadministered, synergistic effects can be observed (13). Since GH responses to GHRH tended to be enhanced in adult GP-Tag Tg, the activation of the GH-IGF-I axis in GP-Tag Tg may be in part due to potentiation of the GH-releasing effect of GHRH. When the mRNA levels of components of GH regulation in pituitary and hypothalamus of GP-Tag Tg mice were investigated, an elevation of the pituitary GHS-R mRNA level was found. It is not clear whether this elevation of GHS-R mRNA in the pituitary contributes to the activated GH-IGF-I axis, since the GH response to ghrelin was not changed in GP-Tag Tg mice. At least these findings indicate that desensitization of GH secretion to ghrelin or downregulation of GHS-R did not occur by chronic elevation of circulating ghrelin in GP-Tag Tg mice.

Adult GP-Tag Tg mice exhibited high glucose level in the basal state and by the glucose tolerance test. Although insulin production was not decreased in the pancreata of GP-Tag Tg mice, insulin secretion after glucose load was significantly attenuated. Since the insulin sensitivity of GP-Tag Tg mice was not reduced, the glucose intorelance in GP-Tag Tg mice was due mainly to the decreased insulin secretion. Given that GP-Tag Tg mice have gastric tumors, there is a possibility that the glucose intorelance is due to the tumors. However, the glucose intolerance observed in malignancy is due mainly to insulin resistance (8, 15), which may be evoked by cytokines (22, 24, 27). Since the glucose intolerance of GP-Tag Tg mice was caused mainly by decreased insulin secretion, it seems not to be the case. It has been reported that acute injection of ghrelin induces suppression of insulin secretion in rodents and humans (6, 30). Our findings suggest that chronic elevation of circulating ghrelin within the physiological range leads to glucose intorelance by suppressing insulin secretion.

There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). Most of the cases did not present

elevated plasma ghrelin levels except for a few cases. A malignant ghrelinoma case reported by Tsolakis et al. (36) showed elevated plasma ghrelin level. This patient maintained his weight despite progression of the tumor, a symptom that might be linked to the elevated ghrelin level. During the clinical course, he developed severe diabetes mellitus, which is consistent with the phenotype of GP-Tag Tg mice. GH and IGF-I levels were normal in this case. A pancreatic ghrelinoma case reported by Corbetta et al. (9) also showed normal GH and IGF-I levels despite elevated plasma ghrelin level. In contrast to these human ghrelinoma cases, GP-Tag Tg mice showed elevated IGF-I levels. The cause of the difference in the GH-IGF-I levels between our mice and these human ghrelinoma cases is unclear. Since the first case mentioned above was a malignant gastric ghrelinoma with liver metastasis, and the second case was of pancreatic origin, plasma ghrelin level might be elevated without any physiological regulation in these cases, although detailed plasma ghrelin level changes were not documented. Considering that the physiological regulation of ghrelin secretion was kept in GP-Tag Tg mice, the circadian rhythm may be needed for ghrelin to keep stimulating the GH-IGF-I axis. Indeed, several reports have shown that chronic treatment of ghrelin attenuates GH response both in vivo and in vitro (35, 39) and that in vitro treatment of pituitary with ghrelin results in decreased GHS-R mRNA levels (21). Further case studies will be required to reveal the relationship between plasma ghrelin levels and the GH-IGF-I axis in human ghrelinoma patients.

The limitation of this study is that the assessment of orexigenic action of ghrelin is difficult in this mouse model since stomach walls of GP-Tag Tg mice gradually become hypertrophic after 9 wk of age, which might affect the feeding behavior. Indeed, GP-Tag Tg mice exhibited decreased food intake and weight reduction despite the elevated plasma ghrelin levels. The hypothalamic mRNA levels of NPY and AgRP, which mediate the orexigenic action of ghrelin (7, 31), were not upregulated in GP-Tag Tg mice. There is a possibility that desensitization of GHS-R to chronic elevated ghrelin may be a cause of the lack of activation of these neurons besides the hypertrophy of the stomach wall. However, hypothalamic mRNA level of GHS-R was not changed. Furthermore, the food intake induced by acute ghrelin administration in GP-Tag Tg mice was comparable with control. These results may not support the idea of desensitization. Leptin and ghrelin have opposing effects on food intake. We examined whether plasma leptin levels of GP-Tag Tg mice were elevated as a compensation for the chronically elevated plasma ghrelin levels, which may cause anorexia. However, the leptin levels were decreased, provably reflecting the decreased fat mass of GP-Tag Tg mice.

In summary, we developed a mouse model of ghrelinoma, GP-Tag Tg mice, in which ghrelin concentrations were significantly elevated in adulthood. These GP-Tag Tg mice exhibited elevated IGF-I levels despite poor nutrition and glucose intolerance due to decreased insulin secretion. These characteristic features of this ghrelinoma mouse could be a guide to diagnose ghrelinoma.

ACKNOWLEDGMENTS

We thank Chieko Ishimoto 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, a research grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and the Takeda Scientific Foundation in Japan.

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Suppression of Experimental Autoimmune Encephalomyelitis by Ghrelin[1]

Michael-Mark Theil,*† Sachiko Miyake,[2]* Miho Mizuno,* Chiharu Tomi,* J. Ludovic Croxford,* Hiroshi Hosoda,‡ Julia Theil,*† Stephan von Hörsten,†§ Hiroaki Yokote,* Asako Chiba,* Youwei Lin,* Shinji Oki,* Takashi Akamizu,¶ Kenji Kangawa,¶ and Takashi Yamamura[2]*

Ghrelin is a recently identified gastric hormone that displays strong growth hormone-releasing activity mediated by the growth hormone secretagogue receptor. While this unique endogenous peptide participates in the regulation of energy homeostasis, increases food intake, and decreases energy expenditure, its ability to inhibit the production of proinflammatory cytokines in vitro indicates its role in the regulation of inflammatory process in vivo. Here we examine the effect of exogenous ghrelin on the development of experimental autoimmune encephalomyelitis (EAE), a representative model of multiple sclerosis. In the C57BL/6 mouse model of EAE induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that alternate-day s.c. injections of ghrelin (5 μg/kg/day) from day 1 to 35 significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting the role of ghrelin as an antiinflammatory hormone. Consistently, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on the new role of ghrelin in the regulation of inflammation with possible implications for management of human diseases. *The Journal of Immunology,* **2009, 183: 2859–2866.**

Small synthetic compounds, referred to as growth hormone (GH)[3] secretagogues (GHS), have been known to stimulate GH release, working through a G protein-coupled receptor called GHS receptor (GHS-R) (1–3). It is now established that a new endogenous peptide, ghrelin, discovered in rat gastric extracts, is an endogenous ligand for GHS-R and is involved in the regulation of GH release. Ghrelin is a 28-aa polypeptide with an essential *n*-octanoyl modification on serine at position 3 (4). Although ghrelin is predominantly secreted from mucosal endocrine cells of stomach, it is widely distributed in various organs, including lymphoid tissues (5, 6). Furthermore, it is measurable in the systemic circulation, indicating its hormonal nature (7).

Ghrelin does not only stimulate GH release, but it also increases food intake, regulates energy homeostasis, and decreases energy expenditure by lowering the catabolism of fat (4, 8, 9). Because of its orexigenic and adipogenic character, ghrelin may be potentially useful for the treatment of anorexia and cachexia (10, 11). Although the precise mechanisms remain to be clarified, the orexigenic activities of ghrelin may be mediated by another feeding regulatory hormone neuropeptide Y (NPY) via stimulation of Y1 and Y5 receptors (12). Furthermore, the antagonistic effect of ghrelin on leptin-induced decrease of food intake seems to be mediated by ghrelin-induced release of NPY and subsequent stimulation of the Y1 receptor (13).

Ghrelin has been shown to exhibit antiinflammatory functions against T cells and macrophages in vitro (14–16). The potential activity of ghrelin as antiinflammatory reagent in vivo was shown in several animal models, including bowel disease (17), arthritis (16, 18), sepsis, and endotoxemia (16, 19, 20). Here we report that s.c. injections of ghrelin could significantly attenuate the clinical severity of the representative model of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice by sensitization against myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide. Furthermore, we demonstrate that in vivo treatment with ghrelin significantly suppressed the mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in microglia and infiltrating T cells derived from the spinal cords of ghrelin-treated mice. Finally, we confirm that LPS-stimulated microglia and monocytes produced lower amounts of proinflammatory cytokines when they were pretreated with ghrelin in vitro. In conclusion, the present study indicates the potential use of ghrelin as an antiinflammatory drug to control human CNS pathology.

Materials and Methods

Mice and reagents

We used female B6 mice (CLEA Japan) between 6 and 10 wk of age in specific pathogen-free conditions. Animal care and use were in accordance

*Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, Tokyo, Japan; †Department of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany: ‡Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka, Japan; §Experimental Therapy, Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany; and ¶Ghrelin Research Project, Translational Research Center, Kyoto University Faculty of Medicine, Kyoto, Japan

Received for publication October 14, 2008. Accepted for publication June 9, 2009.

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[1] This work was supported by the grants from the Ministry of Health, Labor, and Welfare of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and the Ministry of Education, Science, Culture, Sports, and Technology of Japan.

[2] Address correspondence and reprint requests to Dr. Sachiko Miyake or Dr. Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502. E-mail address: miyake@ncnp.go.jp or yamamura@ncnp.go.jp

[3] Abbreviations used in this paper: GH, growth hormone; EAE, experimental autoimmune encephalomyelitis; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; NPY, neuropeptide Y.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803362

Table I. *Amino acid sequence of mouse ghrelin and des-acyl ghrelin*

Peptide	Amino Acid Sequence[a]	Ser^3 acylation	Reference
Ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR	*n*-Octanoicacid	(4)
Des-acyl ghrelin	GSSFLSPEHQKAQQRKESKKPPAKLQPR		(7)

[a] The underlined letter S represents the third serine (Ser^3).

with institutional guidelines. Animal experiments were approved by our institutional review committee. Rat MOG_{35-55} (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Ghrelin and des-acyl ghrelin (Table I) were synthesized as previously described (4, 7).

Immunization and clinical assessment of EAE

We immunized mice (n = 5–15 per group) s.c. in the tail base with 100 μg of MOG_{35-55}-peptide dissolved in 0.1 ml of PBS and 0.1 ml of CFA containing 1 mg of *M. tuberculosis* H37Ra (Difco). Shortly after immunization and 48 h later, the mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories). Clinical scores of EAE were daily assigned as follows: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; 6, death. The cumulative scores were calculated for individual mice by summing up the daily scores.

Administration of ghrelin and des-acyl ghrelin

For EAE treatment, we s.c. injected ghrelin and des-acyl ghrelin diluted in 0.9% saline. In the first series of experiments, mice were injected with ghrelin or des-acyl ghrelin at doses of 0.5, 5, or 50 μg/kg every other day for 35 days. Sham-treated animals were injected with 0.9% saline (standard protocol). In the next experiment, we injected the mice with 5 μg/kg ghrelin every day from day 1 to 10 (induction phase treatment) or from day 11 to 20 (effector phase treatment) and in-between with 0.9% saline. The controls were injected every day from day 1 to 20 with 0.9% saline (alternative protocol).

Assessment of histological EAE

To evaluate the histological manifestations of EAE, we treated mice with 5 μg/kg ghrelin or 0.9% saline following the standard protocol and sacrificed them on day 17 postimmunization. The spinal cords were removed and fixed in buffered formalin. They were embedded in paraffin, sectioned, and stained with H&E and Luxol fast blue for histopathological analysis.

Flow cytometry and isolation of mononuclear cells from the CNS

B6 mice were challenged for EAE, treated following the standard protocol with 5 μg/kg ghrelin or 0.9% saline and sacrificed on day 17 postimmunization. We removed spleen, lymph nodes (LN), and thymus as well as spinal cord from the ghrelin- and saline-treated mice for flow cytometer analysis. Single-cell suspensions were prepared according to standard methods. The spinal cord cell suspensions were centrifuged at 200 × *g* for 10 min and resuspended in 4 ml of 70% isotonic Percoll (Amersham Biosciences)/PBS and overlaid by equal volumes of 37% and 30% isotonic Percoll. The gradient was centrifuged at 500 × *g* for 15 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained for 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-FOXP3, and PerCP-Cy5.5-CD3e mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytofluorometric analysis was performed using a FACSCalibur operated by CellQuest software (BD Biosciences).

Cytokine and cell proliferation assay

MOG_{35-55}-immunized B6 mice were treated s.c. with 5 μg/kg/day of ghrelin or 0.9% saline every day from day 1 to 10. The LN cells were collected on day 11 after immunization and suspended in our standard lymphocyte culture medium (RPMI 1640 supplemented with 5 × 10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin) added with 1% syngeneic mouse serum. The cells were cultured in 96-well round-bottom plates at 1 × 10^6/well for 72 h in the presence of 100 μg/ml MOG_{35-55}. Levels of IFN-γ, IL-17, and IL-4 in the supernatant were determined by using a sandwich ELISA. Proliferative responses were measured using a Beta-1205 counter (Pharmacia) to detect the incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 h of culture.

Evaluation of encephalitogenic T cell induction in B6 mice treated with ghrelin

To evaluate whether in vivo ghrelin treatment may affect the induction of encephalitogenic T cells after immunization with MOG_{35-55}, we evaluated the ability of the lymphoid cells from ghrelin- or saline-treated mice to passively transfer EAE into naive recipients. Donor B6 mice were immunized with MOG_{35-55} and treated every day from day 1 to 10 with 5 μg/kg/day of ghrelin or 0.9% saline. We removed spleens and LN from the donor mice on day 11 and prepared lymphoid cell suspensions. The lymphoid cells were stimulated with MOG_{35-55} (33 μg/ml) in the standard medium added with FCS (10%) for 96 h and then we isolated the $CD4^+$ T cells for cell transfer by depletion of $CD8^+$, $CD19^+$, and $NK1.1^-$ cells. In brief, the MOG_{35-55}-stimulated total lymphoid cells were labeled with PE-CD8a mAb, PE-NK1.1 mAb, and PE-CD19 mAb (BD Pharmingen) for 30 min, washed, and incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min. Using autoMACS (Miltenyi Biotec), we isolated $CD4^+$ T cells ($CD8^-$, $CD19^-$, and $NK1.1^-$ fraction) as a pass-through and suspended the cells in PBS. We injected 1.0 × 10^7 of the cells into the peritoneal cavity of syngeneic recipient mice that had been X-irradiated (550 rad) shortly before. We also injected 200 ng of pertussis toxin i.p. on the same day and 48 h later.

Reverse transcription and real-time PCR

To analyze the mechanism of ghrelin effects in vivo, we extracted total RNA from spinal cord, spleen, thymus, and LN samples using the RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences). Real-time PCR was conducted in the LightCycler quantitative PCR system (Roche Molecular Biochemicals) by using the LightCycler-FastStart DNS Master SYBR Green I kit (Roche Molecular Biochemicals). We followed the manufacturer's specification using 4 mM $MgCl_2$ and 1 pM primers. The primers used are as follows: TNF-α, CTGTGAAGGGAATGGGTGTT (sense) and GGT CACTGTCCCAGCATCTT (antisense); IL-1β, TGAAATGCCACCTTT TGACA (sense) and GTAGCTGCCACAGCTTCTCC (antisense); IL-6, TTCCATCCAGTTGCCTT-CTT (sense) and CAGAATTGCCATTGC ACAAC (antisense); TGF-β, TGCGCTTGCAGA-GATTAAAA (sense) and GCTGAATCGAAAGCCCTGTA (antisense); and HPRT, GTTGGA-TACAGGCCAGACTTTGTTG (sense) and GAGGGTAGGCTGGCCT ATAGGCT (antisense). Values are presented as the relative amount of transcript of each sample normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

In vitro effect of ghrelin on RAW 264.7 monocytes treated with LPS

To examine the effect of ghrelin on monocytes, RAW 264.7 monocytes (American Type Culture Collection) were suspended in the standard culture medium supplemented with 10% FCS and cultured in 96-well flat bottom plates at 1 × 10^5/well overnight. Various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) were added to the culture and 1 h later the cells were stimulated with LPS (Sigma-Aldrich) at various doses (0.1, 1, 10 μg/ml). After 2 h of incubation at 37°C, supernatants were collected and the levels of TNF-α and IL-6 were detected by using a sandwich ELISA.

Isolation of microglial cells from the CNS

The spinal cords were incubated with 35 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals) and 0.1 mg/ml DNaseI (Roche Molecular Biochemicals) in RPMI 1640 medium at 37°C for 30 min. Mononuclear cells were isolated on 30%–80% discontinuous Percoll gradients and were stained with FITC-CD11b mAb, PE-CD45 mAb, and allophycocyanin-CD3 mAb (BD Pharmingen). $CD11b^{high}CD45^{high}$ macrophage cells, $CD11b^{int}CD45^{int}$ microglial cells, and $CD3^+$ T cells were isolated using

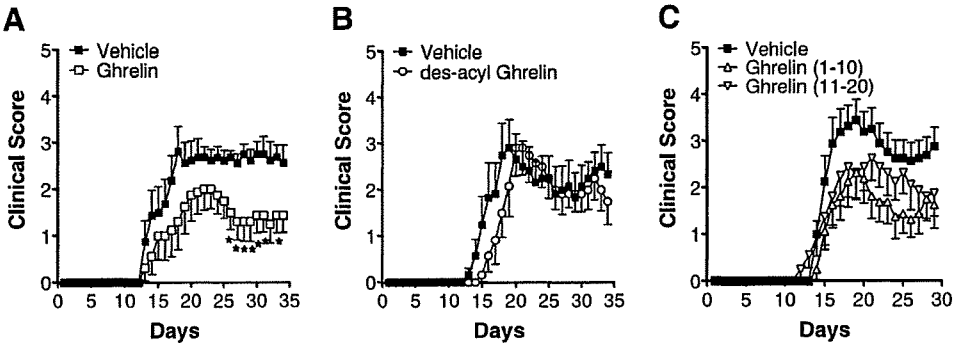

FIGURE 1. Effect of ghrelin on actively induced EAE. EAE was induced in female B6 mice (n = 8 in each group of the three experiments) by immunization with MOG_{35-55}. *A*, The mice were treated every other day starting at the day of immunization with 5 μg/kg ghrelin, while controls were administrated with the vehicle, 0.9% saline, alone. *B*, The mice were injected from day 1 every other day with 5 μg/kg des-acyl ghrelin, whereas controls were subjected to 0.9% saline injections. *C*, Following an alternative protocol, mice were treated from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were treated every day with 0.9% saline injections. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.05$; Mann-Whitney U test).

FACSAria (BD Biosciences). The total RNA was extracted from the isolated cells and was subjected to reverse transcription and real-time PCR.

In vitro effect of ghrelin on microglia cells treated with LPS

Mononuclear cells were prepared from brains of untreated non-EAE mice incubated with Liberase Blendzyme 3 and DNase I as described above and were isolated on 40%–80% discontinuous Percoll gradients. Isolated cells were suspended in DMEM supplemented with 10% FCS and cultured in 96-well flat bottom plates at 2×10^5/well in the presence of ghrelin (10^{-6} M) overnight and later stimulated with LPS at different doses (0.01, 0.1 μg/ml). After 5 h of incubation at 37°C, supernatants were collected and the levels of TNF-α were detected by using a sandwich ELISA.

Statistical analysis

The differences in the clinical score between ghrelin-, des-acyl ghrelin-, and sham-treated groups were analyzed by the nonparametric Mann-Whitney U test. FACS analysis, real-time PCR, ELISA, and proliferation data were subjected to two-way ANOVA. In case of significant differences, a Fisher post hoc test was applied. Probability values of <0.05 were considered as statistically significant.

Results

Ghrelin inhibits EAE

To explore the modulatory effects of ghrelin on inflammatory demyelinating diseases, we employed a model of EAE actively induced in B6 mice with MOG_{35-55}. Although classical forms of EAE are typically characterized by acute paralysis followed by complete recovery, this EAE model shows persistent paralysis with partial recovery as a reflection of persistent inflammatory demyelination in the CNS (21, 22). In the first series of experiments, we injected 0.5, 5, or 50 μg/kg ghrelin to the mice every other day from day 1 to 35 postimmunization, while the control mice were injected with 0.9% saline. The results showed that the continuous injections of 5 μg/kg ghrelin suppressed most efficiently the clinical signs of EAE (Fig. 1*A*), whereas a lower (0.5 μg/kg) or a higher dose (50 μg/kg) showed only a marginal effect (data not shown). The treatment with 5 μg/kg ghrelin did not significantly alter either the onset or peak score of EAE. However, significant differences were noted in mean clinical score after day 25 postimmunization between the ghrelin-treated and the control mice (Fig. 1*A*).

Moreover, the effect of ghrelin on EAE was specific as des-acyl ghrelin, an acyl-modified ghrelin, which lacks the n-octanoic acid on the third serine, and consequently its binding ability to GHS-R (7) (Table I) had no modulatory effect on EAE at any concentration examined (Fig. 1*B* and Table II). Thus, the discrepant results obtained with ghrelin and des-acyl ghrelin indicate that ghrelin treatment would ameliorate the clinical course of EAE via activation of the GHS-R.

To further characterize the effects of ghrelin on EAE, we next examined if treatment lasting for a shorter duration may also be immunomodulatory in vivo. We injected 5 μg/kg ghrelin every day from day 1 to 10 postimmunization (roughly corresponding to the induction phase) or from day 11 to 20 (roughly corresponding to the effector phase). As shown in Fig. 1*C*, both protocols showed similar levels of disease suppression, although it was less notable than the continuous treatment from day 1 to 35 (Table II).

Ghrelin does not influence cellular infiltration into CNS

In the previous results on prophylactic or therapeutic treatment of EAE, clinical suppression of EAE was generally associated with a significant reduction of cellular infiltration in the CNS (23). To clarify if histological manifestation of EAE is also suppressed by ghrelin treatment, we treated MOG_{35-55}-immunized B6 mice with 5 μg/kg ghrelin or 0.9% saline every other day and prepared sections of spinal cords at the peak of disease (day 17 after immunization) (Fig. 2). Clinical signs were milder in the ghrelin-treated mice compared with saline-treated ones. However, histology of the spinal cord sections with H&E staining revealed equivalent levels of cellular infiltration in ghrelin- and saline-treated mice. To confirm this, we isolated mononuclear cells from spinal cords of the

Table II. *Clinical scores of EAE treated with ghrelin or des-acyl ghrelin following different treatment protocols*[a]

Treatment	Incidence	Mean Day of Onset ± SEM	Mean Maximal Score ± SEM	Mean Cumulative Score ± SEM
Vehicle[b]	8/8	16.38 ± 1.13	3.75 ± 0.33	55.44 ± 7.14
Ghrelin[b]	7/8	17.86 ± 1.30	3.29 ± 0.33	36.71 ± 9.99
Vehicle[b]	6/8	18.83 ± 2.55	3.67 ± 0.40	49.33 ± 12.99
Des-acyl ghrelin[b]	6/8	18.00 ± 0.71	3.80 ± 0.44	49.05 ± 8.09
Vehicle[c]	7/8	15.14 ± 0.51	4.43 ± 0.07	50.43 ± 3.10
Ghrelin (1–10)[c]	6/8	16.00 ± 0.73	3.17 ± 0.53	34.00 ± 7.25
Ghrelin (11–20)[c]	7/8	16.29 ± 1.25	3.50 ± 0.45	38.72 ± 8.79

[a] The table shows the results of three separate experiments (n = 8 mice in each group of the three experiments).

[b] After induction of EAE with MOG_{35-55}, mice were treated in two different experiments following the standard protocol of every other day s.c. treatment with 5 μg/kg ghrelin or 5 μg/kg des-acyl ghrelin. The controls were injected with 0.9% saline (vehicle).

[c] Following an alternative protocol, we treated the mice from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9 % saline, while controls were injected every day with 0.9% saline only. Data represent mean ± SEM.

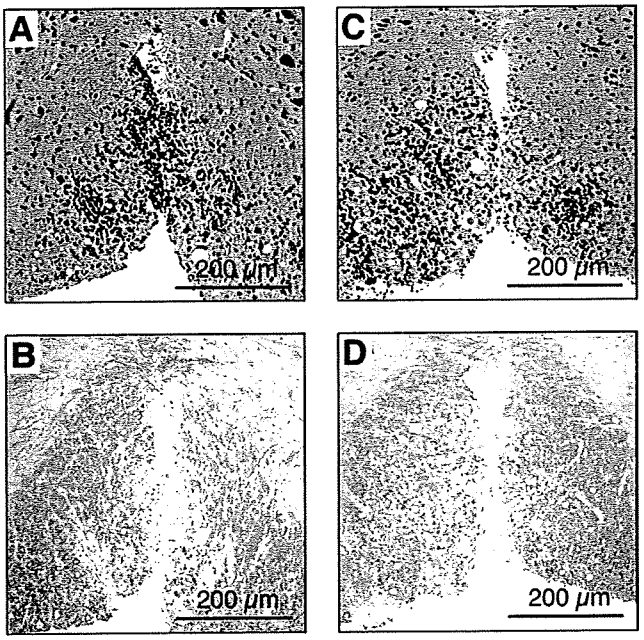

FIGURE 2. Histopathological assessment of the spinal cord of EAE mice. Spinal cords from EAE mice (n = 5/group) were removed on day 17 postimmunization as described in *Material and Methods*. The spinal cord sections from sham- (*A* and *B*) and ghrelin-treated (*C* and *D*) mice were stained in with H&E in the upper panels or Luxol fast blue in the lower ones. Representative sections are shown.

mice at the peak of disease and enumerated the number of the lymphoid cells. Notably, the total cell number was slightly elevated in the ghrelin-treated mice (1.40×10^6/mouse) compared with the saline-treated mice (1.05×10^6/mouse). To further analyze the effects of ghrelin on the formation of CNS inflammation, we evaluated the cellular composition of the CNS-derived lymphocytes by using FACS. Although there was a trend that $CD4^+$ and $CD8^+$ T cell numbers are increased in the lesions of ghrelin-treated mice as compared with saline-treated mice (Fig. 3*A*), it did not reach the level of statistic significance. It was also noted that ghrelin treatment did not alter the number of NK cells

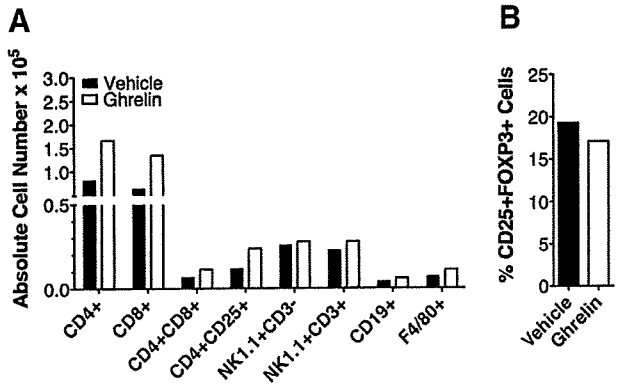

FIGURE 3. Quantification of spinal cord cellular infiltrates by flow cytometry. *A*, The cells were isolated from spinal cords of ghrelin- and sham-treated mice on day 17 postimmunization and subjected to flow cytometer analysis as described in *Materials and Methods*. Data are representative of two independent experiments and presented as absolute cell number (n = 8 mice/group in each experiment). *B*, The proportion of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cord mononuclear cells was analyzed by flow cytometry 20 days after immunization. Data represent two independent experiments (n = 5).

Table III. *Cytokine production and proliferation of MOG_{35-55}-specific T cells after ghrelin treatment*[a]

Treatment	CPM ± SEM	Cytokine Production (pg/ml) INF-γ ± SEM	IL-17 ± SEM	IL-4 ± SEM
Vehicle	47,590 ± 10,988	2,087 ± 487	820 ± 211	ND
Ghrelin	36,663 ± 9,058	2,883 ± 615	674 ± 148	ND

[a] Mice were immunized with MOG_{35-55} and treated with 5 μg/kg ghrelin or 0.9% saline everyday from day 1 to 10 (n = 3/group). Popliteal and inguinal LN cells were harvested on day 11 after immunization and stimulated with 10 μg/ml MOG_{35-55}. CPM marks the proliferative response to MOG_{35-55}. The cytokines were measured in the supernatant by sandwich ELISA after 72 h of stimulation. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. ND, Not detectable.

($NK1.1^+CD3^-$), NKT cells ($NK1.1^+CD3^+$), B cells ($CD19^+$), or macrophages ($F4/80^+$) in the spinal cord lesions. The proportions of $CD25^+FOXP3^+$ cells in the $CD4^+$ T cell population isolated from spinal cords were not altered in ghrelin-treated mice (Fig. 3*B*). In parallel, we also examined the composition of lymphoid cells obtained from spleen, LN, and thymus. Again, we could not reveal any significant change in the subsets of lymphocytes in ghrelin-treated mice (data not shown). Concordant with the histological findings, these data imply that ghrelin did not ameliorate clinical EAE by reducing the numbers of inflammatory cells in the CNS, but rather by regulating the inflammatory potential of the CNS infiltrates.

Ghrelin does not inhibit the induction of MOG_{35-55}-reactive T cells

To elucidate the immunomodulatory mechanism of ghrelin, we examined the cytokine production and proliferative response of draining LN cells to MOG_{35-55} that were obtained from MOG_{35-55}-sensitized mice treated for 10 days every day with ghrelin or saline. The LN cells were collected on day 11 after immunization and stimulated with MOG_{35-55} in vitro. Accordingly, we harvested the supernatant and measured the levels of IFN-γ, IL-17, and IL-4 by using ELISA. Although the IL-4 concentration was under the detection level, IFN-γ and IL-17 could be detected in the MOG_{35-55}-stimulated culture supernatant (Table III). There was no significant difference in the level of IFN-γ and IL-17 when we compared ghrelin-treated and saline-treated groups. Furthermore, ghrelin-treated mice did not differ from saline-treated mice in the proliferative response of the draining LN cells to MOG_{35-55}. We also examined the frequency of $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells in the lymph nodes and spleens using flow cytometry and did not find significant differences between ghrelin-treated and saline-treated mice (data not shown). These results indicate that in vivo ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin does not affect induction of pathogenic autoimmune T cells

To further confirm that MOG_{35-55}-reactive T cells are normally induced in ghrelin-treated mice, we evaluated if the ability of the MOG_{35-55}-sensitized lymphoid cells, obtained from MOG_{35-55}-immunized mice, to transfer EAE into naive mice could be affected by in vivo ghrelin treatment. To this aim, we immunized donor mice with MOG_{35-55} and treated them every day with ghrelin or saline from immunization up to day 10. Next day, we pooled lymphocytes from spleen and LN and cultured them in the presence of MOG_{35-55}. Three days later, $CD4^+$ T cells were purified and injected into recipient mice as described in *Materials and Methods*. It was theoretically possible that in vivo ghrelin treatment does not

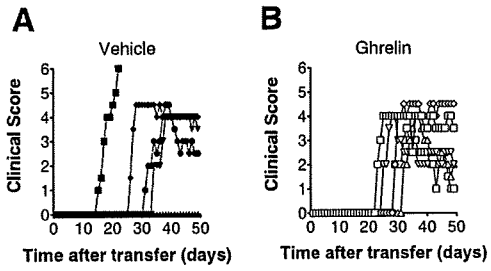

FIGURE 4. Effects of ghrelin treatment on the induction of encephalitogenic T cells. MOG_{35-55}-sensitized lymphoid cells were derived from MOG_{35-55}-immunized and (*A*) saline- or (*B*) ghrelin-treated mice (n = 15/group). The cells were stimulated with MOG_{35-55} and $CD4^+$ T cells were separated 3 days later for passive transfer of EAE into naive mice (n = 5/group). Data represent individual EAE score for each mouse.

inhibit induction of MOG_{35-55}-reactive T cells, but would prohibit the ability to cause EAE in vivo. In postulating that this could happen, $CD4^+$ T cells from ghrelin-treated donors should be less encephalitogenic than those from saline-treated mice. The results showed that transfer of activated $CD4^+$ T cells either derived from saline- or ghrelin-treated donors induced passive EAE in the recipients, showing approximately the same clinical course and severity (Fig. 4). Thus, it can be concluded that ghrelin treatment does not affect the induction of encephalitogenic MOG_{35-55}-reactive $CD4^+$ T cells.

Ghrelin decreases mRNA levels of proinflammatory cytokines in the CNS

After demonstrating that ghrelin does not suppress the infiltration of inflammatory cells in the spinal cord, we wondered whether the cytokine milieu in the ghrelin-treated mice could be significantly altered. To answer the question, we analyzed the mRNA levels of pro- and antiinflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, IL-4, IL-10, and TGF-β) in the spinal cord, spleen, LN, and thymus of ghrelin- and saline-treated mice at the peak of disease (day 17) by using quantitative PCR. Although ghrelin treatment had no effect on the mRNA levels of IL-4, IL-10, and IFN-γ in the spinal cord, spleen, LN, and thymus (data not shown), we found significantly reduced levels of TNF-α ($p < 0.0015$), IL-1β ($p < 0.025$), and IL-6 ($p < 0.025$) in the spinal cord of ghrelin-treated mice, compared with saline-treated ones (Fig. 5*A*). In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. We also found a diminished level of TNF-α mRNA ($p < 0.0001$) in the spleen of ghrelin-treated mice (Fig. 5*B*), whereas we saw no significant change in any of the cytokines that we measured in LN or thymus of ghrelin-treated mice (Fig. 5, *C* and *D*). Because TNF-α, IL-1β, and IL-6 mRNAs were selectively down-regulated in the spinal cord, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. This idea was consistent with the fact that ghrelin treatment did not inhibit the induction of MOG_{35-55}-reactive T cells.

Ghrelin suppresses the proinflammatory cytokine production of LPS-stimulated monocytes

To verify the postulate that in vivo treatment with ghrelin may ameliorate EAE by targeting monocytes, we examined in vitro effects of ghrelin on the monocytic cell line RAW 264.7 that robustly produce proinflammatory cytokines when stimulated with LPS. The RAW 264.7 line cells were first exposed to various doses of ghrelin for 1 h and then stimulated with LPS. We harvested the supernatant 2 h later and measured the levels of TNF-α and IL-6 by ELISA. The results revealed that prior exposure to ghrelin

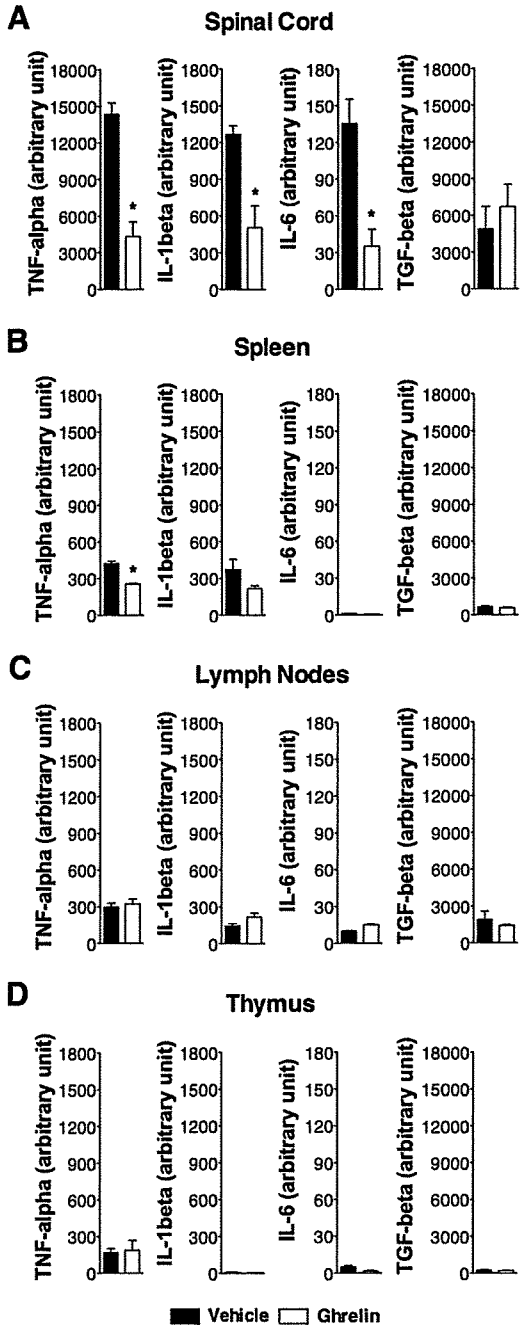

FIGURE 5. Proinflammatory cytokine mRNA expression during EAE in ghrelin-treated mice. Quantitative mRNA expression of proinflammatory cytokines in the spinal cord of MOG_{35-55}-immunized mice subjected to ghrelin or saline treatment on day 17 postimmunization (n = 5/group). Total mRNA was extracted from (*A*) spinal cord, (*B*) spleen, (*C*) LN, and (*D*) thymus. The TNF-α, IL-1β, IL-6, and TGF-β mRNA expression was measured by real-time PCR. Data are presented as relative amount of transcript normalized to HPRT. Data represent mean ± SEM. *, Significant differences between the groups ($p < 0.025$; two-way ANOVA).

would significantly suppress the production of TNF-α ($p < 0.02$) and IL-6 ($p < 0.05$) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effect of ghrelin was very potent, as in addition to the effects on LPS-stimulated monocytes, even the basal production of TNF-α ($p < 0.008$) and IL-6 ($p < 0.03$) was significantly reduced by in vitro ghrelin treatment. Given that in vivo treatment with ghrelin could suppress the

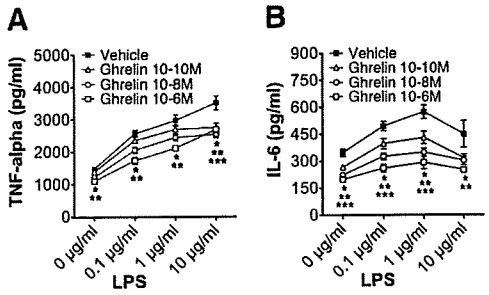

FIGURE 6. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin (10^{-6} M, 10^{-8} M, 10^{-10} M) 1 h before stimulation with 0.1, 1.0, and 10 µg/ml LPS. The (*A*) TNF-α and (*B*) IL-6 production was measured 2 h after LPS stimulation by sandwich ELISA. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. Significant differences at 10^{-6}, 10^{-8}, and 10^{-10} M ($p < 0.05$; two-way ANOVA) are depicted as *, **, and ***, respectively.

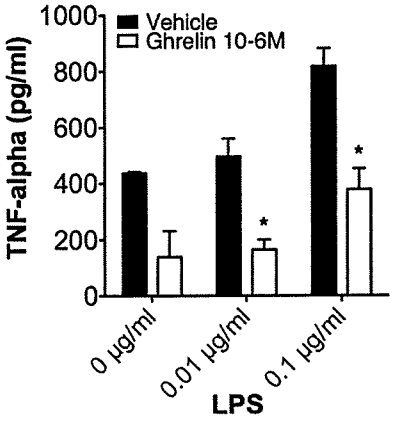

FIGURE 8. Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin (10^{-6} M) overnight and later stimulated with 0.01 and 0.1 µg/ml LPS. Five hours after stimulation, the TNF-α production was measured using ELISA. Data represent mean ± SEM of duplicate samples from one out of two independent experiments. *, Significant differences between the groups ($p < 0.05$; two-way ANOVA).

development of EAE without altering histological EAE or T cell-derived cytokine balance, the ghrelin-mediated suppression of monocyte-produced TNF-α and IL-6 would strongly support the postulate that monocytes are the main target cells in ghrelin-mediated suppression of EAE.

Ghrelin inhibits the expression of proinflammatory cytokines in microglia

The proinflammatory cytokines are known to be produced not only by CNS-infiltrating macrophages but also by T cells and microglia in the course of EAE. To investigate which cells are important in the ghrelin-mediated suppression of EAE, we first examined the expression of proinflammatory cytokines in macrophages. Unexpectedly, the mRNA of IL-1β, IL-6, and TNF-α did not alter in CNS-infiltrating macrophages of ghrelin-treated mice compared with the control mice (Fig. 7*A*). We next examined the expression of these cytokines in other cell types also known as a source of inflammatory cytokines and found reduced expression of these cytokines in microglia (Fig. 7*B*). Additionally, the expression of inflammatory cytokines was decreased in CNS-infiltrating T cells (Fig. 7*C*). Hence, these results suggest that microglia might play a crucial role in ghrelin-mediated inhibition of EAE.

Ghrelin inhibits the proinflammatory cytokine production of LPS-stimulated microglia

We next examined the effect of ghrelin on microglia. To test whether ghrelin directly affects microglia, we isolated mononuclear cells from the brains of untreated mice. In untreated non-EAE mice, most (~77%) of the brain mononuclear cells were $CD11b^+$ cells, and the majority of $CD11b^+$ cells (~95%) were considered as $CD45^{low}$ microglia cells. Among these mononuclear cells, $CD19^+$ B cells were <0.1% and $CD3^+CD45^+$ T cells were 1–1.5%. We cultured the isolated mononuclear cells in the presence of ghrelin overnight and stimulated them with LPS in different doses for 5 h. The TNF-α levels in the culture supernatant were measured by using ELISA. In the presence of ghrelin, the TNF-α levels were significantly reduced (Fig. 8). These results suggest that ghrelin directly affects microglia by reducing the production of inflammatory cytokines.

Discussion

Starvation is known to have immunosuppressive effects (24–26). Although little was known about the mechanistic link between starvation and immunity, recent studies have shed light on the immunomodulatory potency of a range of feeding regulatory hormones such as leptin and NPY. For example, serum leptin is decreased after acute starvation in parallel with immunosuppression or Th2 bias, whereas exogenous leptin would correct the altered Th1/Th2 balance toward Th1 (27, 28). In contrast, NPY is increased after starvation. Exogenous NPY would shift the Th1/Th2 balance toward Th2 and can ameliorate the severity of EAE (29). Interestingly, both peptide hormones are linked to ghrelin in an endocrine feedback system (30). Ghrelin itself is increased after

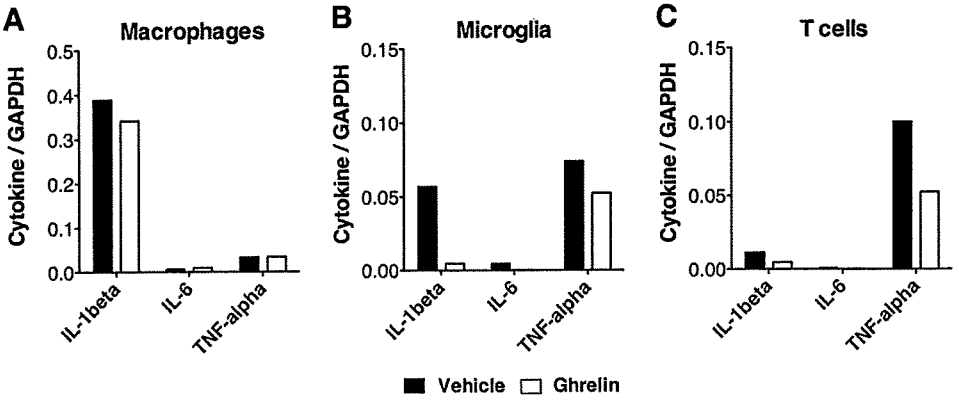

FIGURE 7. Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (*A*) macrophages, (*B*) microglia, and (*C*) T cells obtained on day 20 postimmunization from the spinal cords of MOG_{35-55}-immunized mice treated with ghrelin or saline. The IL-1β, IL-6, and TNF-α mRNA expression levels were measured by real-time PCR. Data are presented as relative amount of transcript normalized to the housekeeping gene GAPDH.

starvation, and it can potently stimulate the release of NPY in the CNS (12). Moreover, ghrelin shows antagonistic effects against leptin (31). Although the available data on the action of ghrelin on leptin or NPY may not be extrapolated to speculate about its role in the immune system, we decided to explore whether ghrelin may exhibit beneficial effects in the modulation of EAE. Furthermore, ghrelin was reported to have protective effects on endotoxic shock in rats (32). Additionally, the wide range of GHS-R expression within the immune cells strongly suggested the immunomodulatory potential of ghrelin (6). Considering its endocrine interactions, ghrelin becomes an interesting candidate for the in vivo modulation of EAE.

To evaluate the effects of ghrelin on the immune system in vivo, we used the representative EAE model induced with MOG_{35-55} in B6 mice. Subcutaneous injections of ghrelin significantly suppressed EAE severity, especially after the peak of disease, while the EAE onset occurred almost similarly in both ghrelin- and sham-treated mice. Priming phase treatment (days 1–10) as well as effector phase treatment (days 11–20) also showed disease-suppressing effects, suggesting a modulatory role of ghrelin during all phases of disease. The unacylated ghrelin form, des-acyl ghrelin, failed to suppress EAE, demonstrating that the disease suppression was mediated by the GHS-R.

The histological findings at day 17 were similar in all animals regardless of the applied treatment. The inflammatory cell infiltration and demyelination occurred in both groups, suggesting a ghrelin effect independent of cell trafficking at the peak of disease. Moreover, we found by FACS analysis that the number of mononuclear cells isolated from the spinal cord and their composition did not significantly alter among ghrelin- and sham-treated mice at the same time point. Our data showed no statistically significant changes in the examined cell subsets, which supported the histological findings of unaffected immune cell traffic to the CNS. This discrepancy between analogous inflammatory status in the spinal cord on the one hand and less severe disease on the other hand in ghrelin-treated mice was remarkable, suggesting cytokine regulation as the possible mechanism of EAE suppression.

Leptin and NPY both influence the Th1/Th2 balance in opposing directions (27–29). Since ghrelin is the most potent NPY-releasing hormone and NPY suppresses EAE by a Th2 bias (29), we examined whether ghrelin affects the Th1/Th2 balance similar to NPY and if its potential mechanism of EAE suppression is primarily mediated on immune cells or secondarily through NPY release. To investigate the effect of ghrelin on the cytokine balance, we measured the cytokine responses of MOG_{35-55}-primed T cells from mice treated with ghrelin or saline. The evaluated IFN-γ, IL-17, and IL-4 levels as well as the proliferative response did not significantly alter between ghrelin- and sham-treated mice. Underlying these observations, we conclude that the suppression of EAE mediated by ghrelin does not affect the T cell-derived cytokine balance. To further address whether ghrelin acts via the NPY pathway, we determined the encephalitogenic potential of $CD4^+$ T cells from ghrelin-treated mice to cause passive EAE in syngeneic recipients. We treated donor animals with ghrelin or saline for 10 days after priming with MOG_{35-55}, and lymphoid cells from the mice were stimulated with MOG_{35-55}. Three days later, $CD4^+$ T cell blasts were isolated and transferred to naive mice. The $CD4^+$ T cells from ghrelin-treated mice did not differ from those from saline-treated mice in the ability to mediate passive EAE, indicating that ghrelin does not primarily affect induction of encephalitogenic $CD4^+$ T cells in vivo. While NPY attenuates EAE by a Th2 bias of encephalitogenic $CD4^+$ T cells (29), our findings likely suggest that ghrelin interacts independently of NPY in the amelioration of EAE.

To further clarify the mechanism of ghrelin-mediated EAE suppression, we examined the mRNA levels of several cytokines of ghrelin- and sham-treated mice at the peak of disease. Our data demonstrate significantly reduced levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the spinal cord and lower levels of TNF-α in the spleen of ghrelin-treated mice. In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. The importance of TNF-α for initiating and sustaining inflammation is well described, as well as its essential role in the development of acute EAE (33, 34). The proinflammatory role of IL-1β and IL-6 in the immunopathology of EAE is also generally accepted (35–38). Thus, the inhibition of TNF-α, IL-1β, and IL-6 must be considered as an important mechanism in the ghrelin-mediated EAE suppression.

Given the selective down-modulation of the proinflammatory cytokines, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. However, the analysis of infiltrating cells and residential microglia revealed that the suppression of proinflammatory cytokines was prominently led by microglia. A decreased expression of these cytokines was also observed in infiltrating T cells. Considering that the transfer of T cells obtained from ghrelin-treated mice induced a similar disease course compared with control mice, the reduction of proinflammatory cytokines in microglia might be important in the ghrelin-mediated suppression of EAE.

In conclusion, the present study demonstrates for the first time to our knowledge that the gastric hormone ghrelin suppresses actively induced EAE by inhibiting production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 with microglia as the main target cells. These findings support an antiinflammatory property of ghrelin, shedding light on its role in immune-endocrine interactions. Consequently, we speculate that ghrelin may serve as an antiinflammatory drug to control human CNS pathology involving the production of proinflammatory cytokines.

Disclosures

The authors have no financial conflicts of interest.

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Endocrine Journal 2009, **56** (9), 1119-1128

NOTE

Ghrelin Increases Hunger and Food Intake in Patients with Restricting-type Anorexia Nervosa: A Pilot Study

MARI HOTTA*, **, RINA OHWADA**, TAKASHI AKAMIZU***, TAMOTSU SHIBASAKI#, KAZUE TAKANO** AND KENJI KANGAWA##

**Health Services Center, National Graduate Institute For Policy Studies, 106-8677 Tokyo, Japan*
*** Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical University, School of Medicine, 162-8666 Tokyo, Japan*
****Ghrelin Research Project, Kyoto University School of Medicine, 606-8507 Kyoto, Japan*
#Department of Physiology, Nippon Medical School, 113-8602 Tokyo, Japan
##National Cardiovascular Center Research Institute, 565-8565 Osaka, Japan

Abstract. Ghrelin increases hunger sensation and food intake in various patients with appetite loss. Anorexia nervosa (AN) begins with psychological stress-induced anorexia and some patients cannot increase their food intake partly because of malnutrition-induced gastrointestinal dysfunction. The effects of ghrelin on appetite, food intake and nutritional parameters in anorexia nervosa (AN) patients were examined. Five female restricting-type AN patients (age: 14-35 y; body mass index: 10.2-14.6 kg/m^2) had persistently complained of gastrointestinal symptoms and failed to increase body weight. They were hospitalized for 26 days (6 days' pre-treatment, 14 days' ghrelin-treatment, and 6 days' post-treatment) and received an intravenous infusion of 3 μg/kg ghrelin twice a day. Ghrelin infusion improved epigastric discomfort or constipation in 4 patients, whose hunger scores evaluated by visual analogue scale questionnaires also increased significantly after ghrelin infusion. Daily energy intake during ghrelin infusion increased by 12-36 % compared with the pre-treatment period. Serum levels of total protein and triglyceride as nutritional parameters significantly increased after ghrelin treatment. There were no serious adverse effects including psychological symptoms. We found that ghrelin decreases gastrointestinal symptoms and increases hunger sensation and daily energy intake without serious adverse events in AN patients. Although the present study had major limitations of the lack of a randomized, placebo-controlled group, non-blindness of the investigators and the small number of patients recruited, it would contribute to further investigations for therapeutic potential of ghrelin in AN patients.

Key words: Ghrelin, Anorexia nervosa, Hunger, Food intake

(*Endocrine Journal* **56**: 1119-1128, 2009)

GHRELIN is mainly secreted by the stomach during starvation and it exerts a potent stimulatory effect on food intake and growth hormone (GH) secretion [1-3]. Endogenous ghrelin and its receptors are involved in the regulation of food intake, adiposity, and GH secretion [4]. Intravenous infusion of ghrelin is reported to increase food intake and body weight in healthy subjects [5-7] and to stimulate appetite and food intake in patients with congestive heart failure [8], chronic obstructive pulmonary disease [9], cancer [10], and functional dyspepsia [11].

Anorexia nervosa (AN) usually begins with psychological stress-induced anorexia and is characterized by fear of weight gain, starvation-induced abnormal behaviors, and a variety of biochemical and endocrinological abnormalities due to malnutrition. Chronic malnutrition induces both functional and organic changes in the gastrointestinal tract [12-14]. Most AN patients complain of chronic or recurrent upper abdominal discomfort and fullness, and chronic constipation. Laboratory examinations of the stomach reveal atrophy of the mucosa, alteration of peristalsis, and de-

Received Jun. 9, 2009; Accepted Aug. 28, 2009 as K09E-168
Released online in J-STAGE as advance publication Sep. 16, 2009
Correspondence to: Mari HOTTA, M.D., Ph.D., Health Services Center, National Graduate Institute For Policy Studies, 7-22-1 Roppongi, Minato-ku, Tokyo 106-8677 Japan.
E-mail: marihs@grips.ac.jp

Table 1. Clinical profile of AN patients in the present study

Case No	1	2	3	4	5
Age on entry (yrs)	27	31	25	35	14
Height (cm)	161	157	156	154	150
Weight before illness (kg) (BMI kg/m^2)	48 (18.5)	48 (19.5)	44.2 (18.2)	50 (21.1)	43 (19.1)
Age of onset (yrs)	16	24	17	20	13
Duration of illness (yrs)	12	6	8	15	1
The minimal weight (kg) (BMI kg/m^2)	29 (11.2)	30 (12.2)	32 (13.1)	23 (9.70)	27.4 (12.2)
Weight on entry (kg) (BMI kg/m^2)	37.9 (14.6)	32.5 (13.2)	35.0 (14.4)	24.2(10.2)	28.2 (12.5)
The increment of daily energy intake (%)	12	36	16	33	14
Weight on the end of study (kg) (BMI kg/m^2)	36.4 (14.0)	31.5 (12.8)	35.7 (14.7)	26.6 (11.2)	28.4 (12.6)
Weight on 6 months after discharge (kg) (BMI kg/m^2)	43 (16.6)	38.5 (15.6)	38.2 (15.7)	28 8 (12.1)	34.5 (15.3)

layed emptying time [15]. Even after becoming fully motivated to gain body weight, AN patients often cannot increase their food intake because of malnutrition-induced gastrointestinal dysfunction, and this delays recovery. Currently prescribed appetite-stimulating drugs such as metoclopramide, cyproheptadine, and sulpiride are not always effective, and any increase in appetite may be minor. Therefore, there is a pressing need for effective appetite-stimulating therapies for AN patients.

To develop a possibly new medical treatment for AN, we investigated the effects of ghrelin on appetite, energy intake, and nutritional parameters in restricting-type AN patients without binge eating/purging as a pilot study.

Subjects and Methods

Subjects

Subjects in the present study comprised 5 Japanese female amenorrheic AN patients aged 26 ± 8 yr (mean ±SD) (range, 14-35 yr) and mean body mass index (BMI) of 13.0 ±1.8 kg/m^2 (range, 10.2-14.6 kg/m^2) (Table 1). Patients met the Diagnostic and Statistical Manual IV (DSM IV) criteria for AN [16], in addition to those of the Survey Committee for Eating Disorders of the Japanese Ministry of Health, Labor and Welfare [17]. All patients had restricting AN, and had never reported binge eating, vomiting or laxative/diuretic abuse. All subjects were tested to be negative for *Helicobacter (H) pylori*. None of the patients had started medication prior to the trial. Four patients except for case 5 had complained of such as epigastric discomfort, abdominal fullness or pain after eating and constipation for several years and had been treated with intensive pshychotherapy as well as supervision of dieticians. All patients had been admitted to undertake hyperalimentation therapy but then lost weight again. They had been motivated to gain weight, but could not increase their food intake, in part because of gastrointestinal discomfort. The study protocol was approved by the institutional review board of Tokyo Women's Medical University. All patients provided written informed consent to participate in this study.

Methods

Study design

Due to ethical reasons, randomized controlled or blind methods were not applied for the present study. Subjects were hospitalized for 26 days (day -6 to day 20) in Tokyo Women's Medical University Hospital (Figure 1). Food intake and subjective hunger sensation were measured for 24 days (day -5 to day 19). The pre-treatment period was defined as the 5 days before ghrelin injection (day -5 to day -1). Subjects received an intravenous infusion of ghrelin (3 μg/kg body weight) for 5 min twice a day (before breakfast and dinner) for 14 days (day 1 to day 14) [11]. After ghrelin infusion, subjects were monitored for the clinical efficacy and safety of ghrelin for 5 days (day 15 to day 19) as a post-treatment period. Since ghrelin at doses of 1 and 5 μg/kg tended to increase appetite dose-dependently and repeated administration of ghrelin at a dose of 3μg/kg increase food intake without severe adverse effects [6, 11], we chose 3μg/kg of ghrelin in the present study.

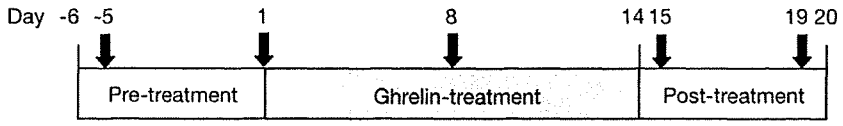

Fig. 1. The timeline of the present study
Subjects were hospitalized for 26 days (day −6 to day 20) and subjective hunger sensation was measured for 24 days (day −5 to day 19). The pre-treatment period was defined as the 5 days before ghrelin injection (day −5 to day −1). Subjects received an intravenous infusion of ghrelin for 14 days (day 1 to day 14). After ghrelin infusion, subjects were monitored for the clinical efficacy and safety of ghrelin for 5 days (day 15 to day 19) as a post-treatment period. Blood and urine samples for biochemical and endocrinological parameters were taken in the morning after overnight fasting and psychological assessment was done on day −5, day 1, day 8, day 15, and day 19, respectively.

Ghrelin used in the present study

Human ghrelin was prepared as previously described [11]. Acylated peptide was dissolved in 3.75 % D-mannitol to yield a final concentration of 180 μg/mL. The solutions were filtered and stored at -20°C in sterile vials. Examination by the Japan Food Research Laboratories (Tokyo, Japan) did not find any traces of endotoxin in the ghrelin solutions. A pyrogen test based on the Pharmacopoeia of Japan was also negative.

Assessment of food intake and attitudes toward food

The primary endpoint of this study was energy intake. Patients were initially served with an amount of food equivalent to their meals at home before hospitalization plus an additional 200 Kcal. Each dish was weighed before and after eating. Energy intake was calculated by dieticians as total energy, carbohydrate, fat, and protein intakes. When subjects ate all of the food served and wanted more, they were allowed to eat self-prepared foods yielding approximately 200 Kcal such as fruit or other snacks. Their attitudes toward food were evaluated by a questionnaire incorporating visual analogue scales (VAS) rating hunger, satiety, prospective consumption, fullness, desire for some meat or fish, desire of something salty, desire of something sweet and desire of something fatty. During pre- and post-treatment, AN patients answered VAS questionnaire at before and after every meal. During ghrelin treatment, they did at 15 min before ghrelin infusion and breakfast or dinner, 15 min after ghrelin infusion before breakfast or dinner, and after those meals. It is demonstrated that food intake correlates with perceptions of hunger and fullness as assessed by VAS in healthy volunteers [18].

Measurement of biochemical and endocrinological parameters

Blood and urine samples for biochemical and endocrinological parameters were taken in the morning after overnight fasting longer than 10 h on day -5, day 1, day 8, day 15, and day 19. Blood samples for ghrelin assay were collected in tubes with 1 mg/mL EDTA-2Na and 500 U/mL aprotinin. They were immediately centrifuged at 4°C, and plasma samples were then acidified with 1 normal HCl and stored at -80°C until assay.

Immunoradiometric assays were utilized to measure levels of plasma GH (Eiken Chemical Co. , Tokyo, Japan) and serum IGF-I (Daiichi Pharmaceutical Co., Tokyo, Japan). Plasma insulin measurements were performed using an ELISA kit (Eiken Chemical Co., Tokyo, Japan). Plasma levels of intact and desoctanoyl ghrelin were measured using Active Ghrelin and Desacyl-Ghrelin ELISA kits (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively.

Psychological assessment

Depression and anxiety levels were evaluated using the Japanese versions of the self-rating depression scale (SDS) [19] and state-trait anxiety inventory (STAI) [20] on day -5, day 1, day 8, day 15 and day 19, respectively. Eating behaviors, weight, and body image concerns were also assessed by eating disorder inventory (EDI) [21] on the same time as described.

Statistics

Data are expressed as mean ± SE. Two-way analysis of variance (ANOVA) was used for energy and nutrient intakes and for biochemical and endocrinologic data. Appetite scores were analyzed by a Wilcoxon

signed rank test comparing the changes in VAS. Statistical analyses were performed using the computer statistical package SPSS (version 13.0.; SPSS Inc., Chicago, IL). Levels of significance were determined at $p<0.05$.

Results

Gastrointestinal symptoms and hunger sensation

After ghrelin injection, all patients except for case 2 reported that they had sensations of stomach activity or that their upper abdominal fullness disappeared. Borborygmi were frequently audible just after each ghrelin infusion in all patients. During ghrelin treatment, no patients reported constipation. As case 5 complained of loose stools, the dose of ghrelin was reduced to 1.5 μg/body weight from day 7 to day 14 and this improved her symptoms.

Hunger sensation evaluated by VAS was higher just after ghrelin infusion than that before ghrelin infusion in all patients except for case 2 (Figure 2). The stimulatory effects of ghrelin on hunger sensation disappeared after eating and did not last until next meal. Only in case 1, hunger scores before breakfast or dinner during ghrelin treatment were lower than those during both the pre- and post-treatment periods.

Food intake and body weight

The mean daily intakes of energy, carbohydrate, fat and protein are presented in Figure 3. The daily energy intake of the 5 patients during the pre-treatment period ranged from 825 to 1426 Kcal. During ghrelin infusion, all patients except for case 5 showed a statistically significant increase in daily energy intake. The mean increase in daily energy intake during ghrelin infusion was 20 ± 4 % when compared with the pre-treatment period. The mean food intake during ghrelin treatment in case 2, who did not report an increase in hunger sensation after ghrelin injection, significantly increased compared to that of pre-treatment. Analysis of nutrients revealed significant increases in daily intakes of carbohydrate (in 3 patients; cases 2, 3, and 4), fat (in 1 patient; case 4) and protein (in all patients). During the post-treatment period, daily energy, carbohydrate and protein intakes remained higher than those in the pre-treatment period in 3 patients (cases 2, 3, and 4). The daily fat intake during post-treatment period also remained higher than that in the pre-treatment period in 4 patients (cases 2, 3, 4, and 5). The increments of body weight in 5 patients were ranged from -1.5 to 2.4 kg during the ghrelin study (Table 1). Case 4 increased water and fat components evaluated by dual X-ray absorptiometry (data not shown).

Biochemical and endocrinological changes

Complete blood count did not change significantly during this study. Serum total protein and triglyceride levels significantly increased after ghrelin treatment (Table 2). Other nutritional markers including serum levels of transferrin and glucose showed a tendency to increase during and after ghrelin treatment, but this did not reach statistical significance. With the exception of case 4, in whom elevated transaminase levels due to malnutrition were improved by ghrelin treatment, liver function was stable over the study period.

Mean plasma levels of insulin and leptin did not increase significantly during ghrelin treatment. Although the elevated plasma level of GH decreased and the suppressed serum level of IGF-I improved during the study in case 4, other patients did not show a significant change in those parameters. Mean plasma levels of PRL and ACTH measured in the morning before ghrelin injection did not change significantly during ghrelin treatment.

We previously reported mean levels of plasma active and desacyl ghrelin in healthy young women as 29.9 ± 3.1 and 94.1 ± 7.5 pmol/L, respectively [22]. In the present study, the plasma levels of active ghrelin in AN patients ranged from 13 to 73 pmol/L (mean, 42) before ghrelin treatment and then did not show a significant change. Plasma levels of desacyl ghrelin in AN patients ranged from 80 to 731 pmol/L (mean, 280) before treatment, and then showed a tendency to decrease during ghrelin treatment.

Adverse effects

No serious adverse events occurred in all cases during ghrelin treatment. We did not detect any changes in vital signs or biochemical and endocrinologic data after ghrelin treatment. The only exceptions were loose stools in case 5 and an occasional warm sensation in the trunk or mild sweating in 2 subjects. No patients developed somnolence during ghrelin treat-

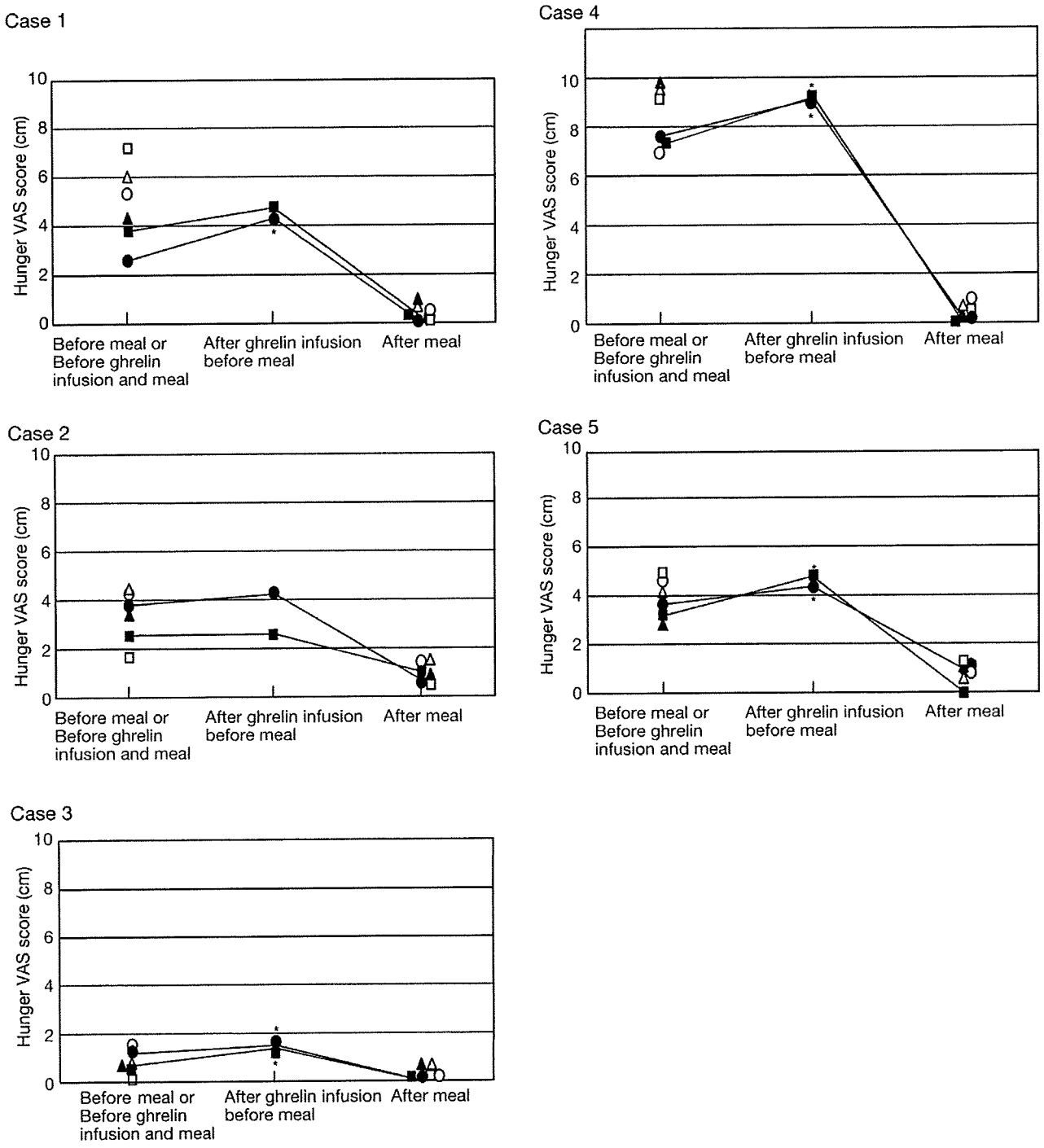

Fig. 2. Changes in hunger evaluated by VAS in AN patients

During pre- and post-treatment, AN patients answered VAS questionnaire at before and after every meal. During ghrelin treatment, they did at 15 min before ghrelin infusion, 15 min after ghrelin infusion before breakfast or dinner, and after those meals. Open circles (○), triangles (△) and squares (□) represent the mean of VAS hunger scores for breakfast, lunch, and dinner during pre and post-treatment periods, respectively. Closed circles (●), triangles (▲) and squares (■) represent the mean of VAS hunger scores for breakfast, lunch, and dinner during ghrelin treatment, respectively.

Data are expressed as mean. * $p<0.05$ *vs.* before ghrelin infusion

The mean of hunger scores before breakfast or dinner evaluated by VAS significantly increased after ghrelin infusion in all cases except for case 2.

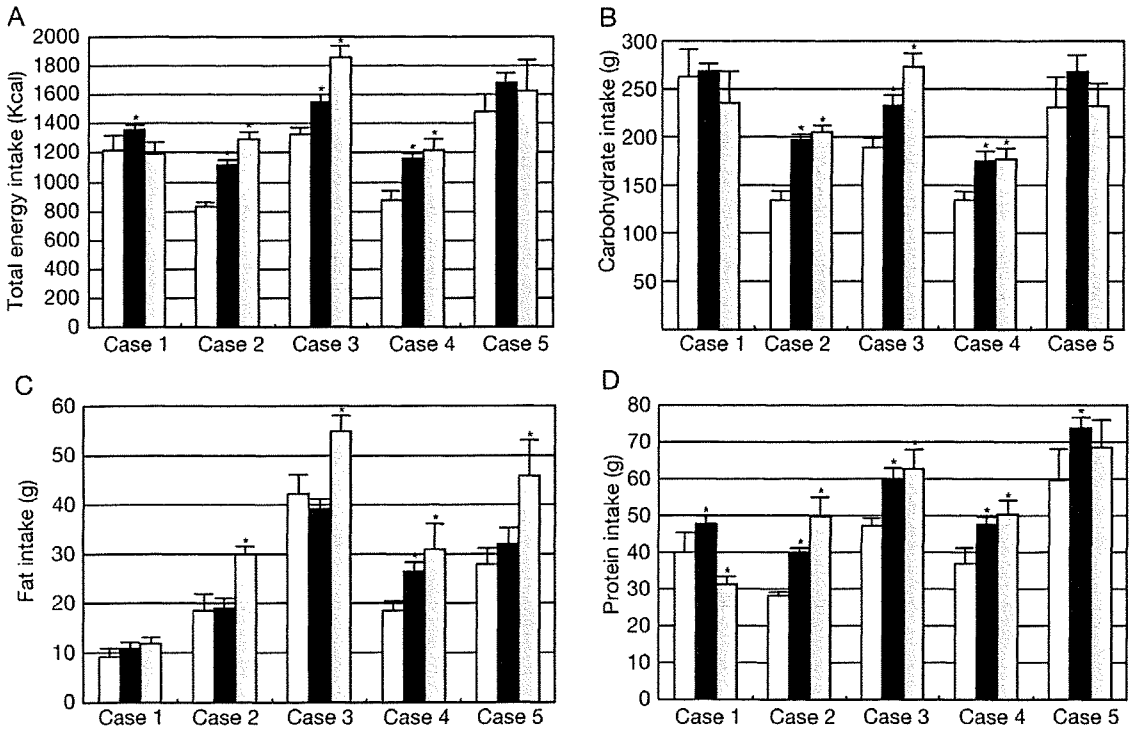

Fig. 3 Changes in the mean of total energy (panel A), carbohydrate (panel B), fat (panel C), and protein (panel D) intakes of AN patients.

Open (□), closed (■) and grey (◪) bars represent the mean of intake during pre-treatment, ghrelin treatment, and post-treatment periods, respectively. Data are expressed as mean ± SE. * $p<0.05$ *vs.* pre-treatment period.

Across the 5 patients, mean increase in daily energy intake during ghrelin infusion was 12-36%. Energy intake in the post-treatment period remained higher than that in the pre-treatment period in 3 patients.

Table 2. Changes in biochemical and endocrinological data in AN patients during the present study

	Day 1	Day 8	Day 15	Day 19
White blood cell (/μL)	3200±230	2820±331	2660±388	3240±614
Hemoglobin (g/dL)	12.7±0.8	13.0±1.2	12.7±1.1	13.0±0.9
Platelet (x10^4/μL)	15.8±2.4	16.0±2.4	15.6±1.9	16.4±1.8
Total protein (g/dL)	6.5±0.4	6.9±0.1	6.8±0.4	7.1±0.3*
Transferrin (mg/dL)	179±22	195±21	196±15	208±10
Retinol binding protein (mg/dL)	3.0±0.3	3.0±0.4	3.1±0.3	3.1±0.2
Blood sugar (mg/dL)	75±5	79±2	81±2	81±2
AST(U/L)	86±58	32±7	27±2	31±3
ALT(U/L)	164±139	60±35	37±11	38±10
Cholinesterase (U/L)	220±29	220±30	214±28	216±25
Triglyceride (mg/dL)	47±10	80±15*	72±9*	83±10*
Total cholesterol (mg/dL)	179±23	187±23	170±24	182±18
Immunoreactive insulin (U/mL)	2.00±0.29	1.57±0.46	2.21±0.46	2.39±0.27
Leptin (ng/mL)	1.4±0.3	1.2±0.2	1.3±0.1	1.3±0.1
GH (ng/mL)	16.6±14.6	11.2±10.3	8.7±7.0	3.6±1.9
IGF-I (ng/mL)	115±37	116±28	128±32	123±35
PRL (ng/mL)	10.8±2.0	8.2±1.3	9.9±1.5	9.3±1.5
ACTH (pg/mL)	28.3±7.4	19.1±4.3	22.9±2.6	25.1±3.5
Active ghrelin (pmol/L)	42±19	45±12	54±15	46±9
Desacyl ghrelin (pmol/L)	280±115	198±26	206±37	198±37

Data are expressed as mean ± SE. *$p<0.05$ compared to day 1.

ment. In terms of psychological tests, SDS and STAI showed no significant change during the study, and EDI did not show any increased fear of weight gain in these patients (data not shown).

Clinical course after discharge

All patients gained weight after discharge, as shown in Table 1. In case 3, menstruation resumed 6 months after discharge.

Discussion

The present study showed that ghrelin infusion (3 μg/kg twice a day) can decrease gastrointestinal symptoms and enhance hunger sensation and daily energy intake without serious adverse events in restricting-type AN patients. The major limitations of the present study relate to the lack of a randomized, placebo-controlled group and non-blindness of the investigators and the small number of patients recruited. A non-treated group is not possible due to ethical reasons. Although non-ghrelin infused subjects who receive intense counseling and supervision of dietitian might be considered as a control group, all subjects in the present study had already received those treatments as well as total parenteral nutrition during the previous admission but failed to increase body weight due to gastrointestinal symptoms. Since the daily energy intake of post-treatment period was still higher than that of pre-treatment period, we could not exclude a placebo effect of ghrelin. However, we insist that 4 patients who failed in gaining body weight for long periods but they could increase their food intake during and after ghrelin infusion. It is speculated as the patients told us that ghrelin triggered an improvement in gastrointestinal function, which ameliorated the fear of gastrointestinal discomfort after eating in these patients.

Ghrelin seems to improve gastrointestinal motility in AN patients in the present study. It is notable that borborygmi occurred immediately after ghrelin infusion and that abdominal fullness or constipation disappeared in all patients. Ghrelin plays a role in the regulation of gastrointestinal motility and acid secretion in rats [23-25] and increases the gastric emptying rate in normal-weight humans [26]. Although we did not investigate gastric emptying rate in AN patients after ghrelin injection, ghrelin improved epigastric discomfort. This was probably mediated partly through increased gastric peristalsis as shown in other diseases with gastrointestinal dysfunction [27-30].

Ghrelin infusion increased hunger scores evaluated by VAS questionnaires of AN patients in the present study. Although AN patients often report not to feel hunger or satiety sensation, hunger scores was higher just after ghrelin infusion than that before ghrelin infusion in 4 patients. Since the sensation of hunger is usually correlated with gastric emptying in humans [31], enhanced hunger sensation in AN patients may be caused in part by ghrelin-induced gastric motility. However, the stimulatory effects of ghrelin on hunger score did not last until the next meal. We considered that the short-term effect of ghrelin on hunger sensation is related to its rapid degradation. The plasma concentration of ghrelin reaches the peak at 15 min after injection and rapidly decreases [6]. Hunger scores before breakfast or dinner during ghrelin treatment were lower than those during both the pre- and post-treatment periods in case 1. It is likely that abdominal fullness induced by the increased amount of food eaten in the foregoing meal during ghrelin treatment probably disturbed the hunger sensation on the next meal.

In previous reports, continuous or repeated ghrelin infusion increased hunger sensation and food intake in healthy volunteers and various patients with appetite loss. Ghrelin infusion at a dose of 5 pmol/kg/min for 270 min increased food intake by 28 % in healthy young Caucasian volunteers [5] and by 31 % in middle-aged and elderly cancer patients [10]. Ghrelin infusion (2 μg/kg twice a day) for 3 weeks increased food intake and body weight by 0.8 kg in elderly patients with congestive heart failure [9], and by 1 kg in elderly patients with chronic obstructive pulmonary disease [8]. Moreover, in patients with functional dyspepsia, ghrelin infusion (3 μg/kg twice a day) for 2 weeks increased hunger sensation and food intake by 29 % without significant weight gain [11]. Since 1 kg weight gain requires 7000-8000 Kcal, the increase in energy intake achieved for 14 days in this study was not enough to lead to any considerable weight gain. Although case 4 gained 2.4 kg and showed remarkable improvement in nutritional parameters and malnutrition-related liver dysfunction, we believe that water retention during the refeeding period contributed to this weight gain [32]. A decrease in body weight of 2 patients (cases 1 and 2) during ghrelin study might be attributable to a decrease in malnutrition-induced fluid