

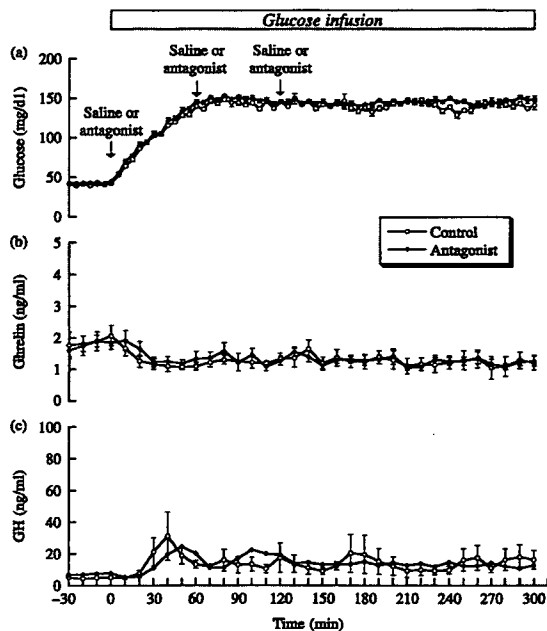
**Results**

In the HGC (Experiments 1 and 2), plasma glucose concentrations were clamped at 100 mg/100 ml above the initial level between 60 and 300 min after the start of glucose infusion (Figs 1a and 3a). There was no difference in basal plasma glucose concentrations before glucose infusion between the fasting (Fig. 1a) and meal-fed state (Fig. 3a).

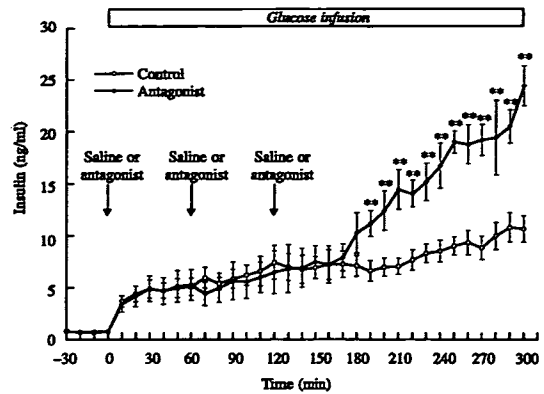
In the fasting state (Experiment 1), average plasma ghrelin level was 1.8 ng/ml before glucose infusion (Fig. 1b). Plasma ghrelin levels were significantly ( $P < 0.01$ ) decreased after the commencement of glucose infusion in both groups (Fig. 1b). Plasma GH levels were significantly ( $P < 0.05$ ) increased after glucose infusion in both groups (Fig. 1c). Plasma ghrelin and GH levels were not affected by the ghrelin antagonist (Fig. 1b and c).

Changes in plasma insulin levels in the fasting state are presented in Fig. 2. Plasma insulin levels were significantly ( $P < 0.01$ ) increased by glucose infusion in both groups. There was a greater incremental increase ( $P < 0.01$ ) in plasma insulin concentrations in the D-Lys3-GHRP6 group when compared with the control beginning about 60 min after the third administration of the antagonist.

In the meal-fed state (Experiment 2), average plasma ghrelin level was 0.5 ng/ml before glucose infusion (Fig. 3b). Plasma ghrelin levels significantly ( $P < 0.01$ ) increased and reached a plateau within 10 min after the commencement of ghrelin

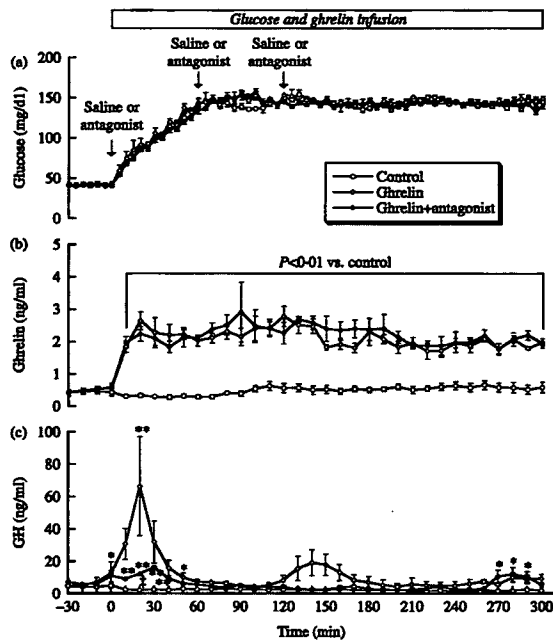


**Figure 1** (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  s.e.m. ( $n = 4$ ).

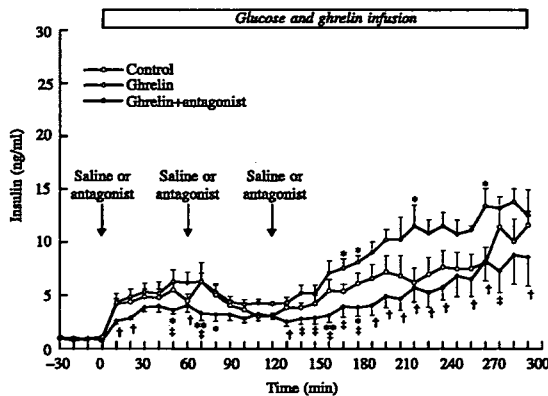


**Figure 2** Average insulin plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  s.e.m. ( $n = 4$ ). \*\* $P < 0.01$  versus control.

infusion (Fig. 3b). There were temporal increases ( $P < 0.05$ ) in plasma GH levels during ghrelin infusion (Fig. 3c). Increase in plasma GH levels was significantly ( $P < 0.01$ ) depressed by the ghrelin antagonist between the first- and second administration of the ghrelin antagonist (Fig. 3c).



**Figure 3** (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0.04  $\mu$ g/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  s.e.m. ( $n = 4$ ). \* $P < 0.05$  versus control, \*\* $P < 0.01$  versus control, \* $P < 0.01$  versus ghrelin.



**Figure 4** Average insulin plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0.04 µg/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-Lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  S.E.M. ( $n=4$ ). \* $P<0.05$  versus control, \*\* $P<0.01$  versus control, <sup>†</sup> $P<0.05$  versus ghrelin, \* $P<0.01$  versus ghrelin.

Changes in plasma insulin levels in the meal-fed state are presented in Fig. 4. Plasma insulin levels were significantly ( $P<0.01$ ) increased by glucose infusion in all groups. There was a biphasic insulin increment in control and ghrelin-infused group. Ghrelin significantly ( $P<0.05$ ) enhanced only the second phase of insulin secretion. The ghrelin antagonist significantly ( $P<0.05$ ) depressed both the first- and second phase of insulin secretion.

## Discussion

Effects of ghrelin on insulin secretion are bi-directional, since ghrelin exerts both stimulatory and inhibitory effects on insulin secretion. Ghrelin inhibits insulin secretion in fasted humans (Broglia *et al.* 2001) and mice (Reimer *et al.* 2003). By contrast, i.v. injection of ghrelin accelerates insulin secretion in free-feeding rats (Lee *et al.* 2002) and fed sheep (Takahashi *et al.* 2006). Ghrelin stimulates insulin secretion from isolated pancreatic islets of freely fed rats (Date *et al.* 2002). Taken together, these reports suggest that ghrelin differentially modulates insulin secretion, dependent on feeding states. The present study has clearly demonstrated that ghrelin inhibits and stimulates glucose-induced insulin secretion in fasting- and meal-fed state respectively.

Baseline of plasma ghrelin fluctuates between 0.5 and 2.0 ng/ml depending on the feeding states in sheep. The fluctuation of plasma ghrelin makes it difficult for us to determine the effects of ghrelin, since it is uncertain which level of plasma ghrelin is most effective on insulin secretion in different feeding states. In order to determine physiological effects of ghrelin as far as possible, we avoided administering ghrelin to the fasting animals in which plasma ghrelin levels

had reached plateau (2.0 ng/ml), while we did not administer the ghrelin antagonist alone to the fed animals in which plasma ghrelin levels had reached nadir (0.5 ng/ml). Our previous study showed that ghrelin significantly enhanced glucose-stimulated insulin secretion at 1.0 ng/ml of plasma level in the meal-fed state (Takahashi *et al.* 2006). Therefore, the effect of the antagonist on insulin response at plasma ghrelin levels between 1.0 and 1.5 ng/ml during the HGC in the fasting states would be comparable with the effect of ghrelin administration in the meal-fed state.

In the meal-fed state, exogenous ghrelin enhanced glucose-induced insulin secretion as shown previously (Takahashi *et al.* 2006). The enhancement by ghrelin of glucose-stimulated insulin secretion was delayed in the present study when compared with the previous study. This may be related to weak insulin response to glucose in the animals newly applied to the HGC. Repeated doses of a ghrelin antagonist, D-Lys3-GHRP-6, counteracted the stimulatory effect of exogenous ghrelin on insulin secretion, suggesting that ghrelin stimulates insulin secretion via GHS-R1a in the meal-fed state. In addition, even lower insulin secretion by ghrelin plus antagonist compared with control suggests the possibility that basal ghrelin in the meal-fed state may also enhance insulin secretion.

In the fasting state, repeated doses of D-Lys3-GHRP-6 significantly enhanced glucose-induced insulin release, suggesting that endogenous ghrelin suppresses insulin secretion in the fasting state. The present result is supported by the finding that GHS-R antagonist enhances glucose-induced insulin release from perfused rat pancreas and increases plasma insulin levels in rats (Dezaki *et al.* 2006). In the present study, the insulin secretory response to the ghrelin antagonist emerged 60 min after the last dose of the drug. The hypoglycemic effect of the ghrelin antagonist emerges 2 days after the commencement of daily injections in diabetic mice where plasma ghrelin levels are high (Dong *et al.* 2006). In the fasting state, therefore, high levels of circulating ghrelin might compete with the ghrelin antagonist for GHS receptors, thus delaying the action of the drug.

Ghrelin stimulates GH secretion in sheep (Iqbal *et al.* 2006, Takahashi *et al.* 2006). In the present study, therefore, plasma GH concentrations were measured as an indicator for ghrelin receptor blockade. In the fed-state, D-Lys3-GHRP-6 attenuated ghrelin-induced GH secretion, indicating that the antagonist certainly blocked the ghrelin receptors. In the fasting state, however, the antagonist did not affect GH secretion, probably because GHS-R1a might not be involved in GH elevation after glucose infusion. Furthermore, ghrelin receptor mRNA expression is upregulated by fasting in the hypothalamus and pituitary (Kim *et al.* 2003). This may be related to the ineffectiveness of the ghrelin antagonist in GH suppression in the fasting state.

In conclusion, ghrelin inhibits and stimulates glucose-induced insulin secretion via GHS-R1a in fasting- and meal-fed state respectively. Further studies are required to identify

underlying mechanisms for alteration in the effects of ghrelin on insulin secretion in different feeding states.

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## Neuromedin S exerts an antidiuretic action in rats

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### Abstract

We recently identified neuromedin S (NMS) as an endogenous ligand for the FM-4/TGR-1 receptor. Here, we examined the possible involvement of central NMS in regulation of urinary output and vasopressin (AVP) release in rats. Intracerebroventricular (icv) injection of NMS induced a dose-dependent increase in the plasma level of AVP, followed by a decrease of nocturnal urinary output. Expression of cFos after icv injection of NMS was observed in the suprachiasmatic nucleus (SCN), arcuate nucleus, paraventricular nucleus (PVN), and supraoptic nucleus (SON). The cFos expressing cells in PVN and SON, but not SCN, were then double-stained using antibodies against the vasopressin. On the other hand, icv injection of neuromedin U, which also binds to the FM-4/TGR-1 receptor, required a concentration ten times higher than that of NMS in order to exert the same antidiuretic potency. These results suggest that central NMS may exert a physiological antidiuretic action via vasopressin release.

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**Keywords:** Neuromedin S; Neuromedin U; Vasopressin; Antidiuretic action

In addition to neuromedin U, we have recently discovered the novel peptide neuromedin S (NMS) as an endogenous ligand for two orphan G-protein-coupled receptors, FM-3/GPR66 and FM-4/TGR-1, using a reverse-pharmacological technique [1]. FM-3/GPR66 and FM-4/TGR-1 had already been identified as neuromedin U receptor type-1 (NMUR1) and type-2 (NMUR2), respectively [2–4]. Rat NMS is a 36-amino acid neuropeptide that is specifically expressed in the suprachiasmatic nucleus (SCN) [1]. Although NMS shares a C-terminal core structure (7 amino acid residues) with NMU and activates both recombinant NMUR1 and NMUR2 expressed in Chinese hamster ovary cells, NMS is not a splice variant of neuromedin U, because both the NMS and neuromedin U genes have been mapped to discrete chromosomes. In

addition, although neuromedin U mRNA has been detected in peripheral and central organs, the distribution of NMS is limited to the testis, spleen, and hypothalamus, especially the SCN [1–3]. NMS has been shown to have several physiological roles in rats, including involvement in circadian oscillation systems, since intracerebroventricular (icv) administration of NMS induces phase-dependent phase shifts in the circadian rhythm of locomotor activity in rats kept under constant darkness [1]. In addition, NMS may be involved in feeding regulation, because icv injection of NMS decreases food intake in a dose-dependent manner [5]. Recently, it has been shown that NMS regulate luteinizing hormone secretion [6].

NMUR1 is located in a wide range of peripheral tissues such as intestine, testis, pancreas, uterus, lung, and kidney. On the other hand, expression of NMUR2 is limited to areas of the central nervous system [2,3]. Therefore, NMS may have an unknown role in the central nervous system. We examined the expression of cFos after icv injection of

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NMS to search for the site of action of NMS in the central nervous system. When NMS was injected icv, cFos expression was observed specifically in the paraventricular nucleus (PVN), arcuate nucleus (Arc), supraoptic nucleus (SON), and SCN [5]. Although the expression of cFos in the PVN, Arc, and SCN may be related to its biological role in feeding regulation and circadian rhythm mentioned above, its physiological role in the SON is unknown.

On the other hand, the PVN and SON are known to be common sites synthesizing arginine-vasopressin (AVP) and oxytocin (OXT) [7,8]. Recently, NMUR2 was detected in AVP-positive neurons in the PVN, indicating a possible role of NMS in the secretion of this hormone [9]. However, the relationship between NMS and AVP remains to be elucidated. AVP synthesized in the magnocellular region of the SON and PVN is exported to the posterior pituitary gland and secreted into the peripheral blood, subsequently acting on the kidney through specific receptors in the distal renal tubule to decrease urine volume. In the present study, we examined the possible involvement of central NMS in AVP secretion and urinary output in rats.

## Materials and methods

**Animals and icv injection.** Male Wistar rats, weighing 300–350 g, were housed in individual Plexiglas cages in an animal room maintained under a constant light–dark cycle (lights on from 7:00 to 19:00 h) and temperature ( $22 \pm 1$  °C) for at least one week. Food and water were provided ad libitum. Icv cannulae were implanted into the lateral cerebral ventricles by a method that has been described previously [5]. After surgery, all rats were housed individually in Plexiglas cages. During a 6-day postoperative recovery period, the rats became accustomed to the handling procedure. Rat NMS or rat neuromedin U (Peptide Institute Inc., Osaka, Japan) was dissolved in saline, and 10  $\mu$ l of the solution was injected into each free-moving rat through a 27-gauge injection cannula connected to a 50- $\mu$ l Hamilton syringe. All procedures were performed in accordance with the Japan Physiological Society's guidelines for animal care.

**Measurement of plasma AVP.** Whole blood was collected by decapitation at 5, 60, and 180 min after icv injection of 0.02, 0.2, and 2 nmol NMS at 18:45 h into a tube containing EDTA and the proteinase inhibitor, Aprotinin (Sigma–Aldrich Inc.). Each group consisted of 8 rats. After centrifugation at 4 °C, the plasma was stored at  $-80$  °C until measurement of AVP. AVP concentration was measured using an EIA kit (Assay designs Co., Ann Arbor, MI, USA) following the manufacturer's protocol.

**Measurement of urinary volume and water intake.** Before measurement, rats ( $n = 4$ /each group) were maintained individually in metabolic cages for four days to allow them to habituate. Twelve-hour urinary volume and water intake were measured every day in the dark phase, because each was very slight during the light period. After habituation, icv injection of 0.02, 0.2, and 2 nmol of NMS and neuromedin U was performed at 18:45 h, and then urinary volume and water intake were measured at 07:00 on the following morning. The control rats were injected with the same volume of saline. These experiments were repeated twice, and a total of 8 samples were collected in each group.

**Immunohistochemistry for FM-4.** Immunohistochemical analyses for the NMUR2 in the PVN and SON were performed using a modification of a method described previously [10]. The brain was placed in fixative for 5 days at 4 °C and then transferred to 0.1 M phosphate buffer containing 20% sucrose. Each brain was cut into serial, 18- $\mu$ m-thick sections at  $-20$  °C with a cryostat. The sections were incubated for 2 days with a rabbit anti-NMUR2 antibody (Abcam Ltd., Cambridge, UK) at 4 °C. Slides were then incubated with Alexa-546-labeled goat–anti-rabbit IgG

antibody (Molecular Probes Inc., OR, USA, dilution 1:400). Samples were observed with the aid of an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan).

**RT-PCR of FM-3 and FM-4 mRNA in the PVN and SON.** The PVN and SON were punched out from the frozen brain slices using a method described previously [11]. Spinal cord was used as a control tissue, because it shows abundant expression of NMUR1 and NMUR2 [12]. Total RNA was extracted from the samples using Trizol reagent (Invitrogen Co., Carlsbad, CA) as described previously [13]. First-strand cDNA was synthesized from 2  $\mu$ g of total RNA by random primer reverse transcription using a SuperScript III First-strand cDNA synthesis kit (Invitrogen Co.). The resulting cDNA was subjected to PCR amplification using sense and antisense primers specific for FM-3 and FM-4 by using iCycler (582BR; Bio-Rad Laboratories, Tokyo, Japan). The primer sets used for rat NMUR1 and NMUR2 were as follows: rat NMUR1 primer set: 5'-C ACGACTCCCATAGCCA-3' (sense), 5'-TCACACCTGGATCCCT GTT-3' (antisense); rat NMUR2 primer set: 5'-GATGAATCCCTT GAGGCGAA-3' (sense), 5'-ATGGCAAACACGAGGACCAA-3' (antisense). PCR products were electrophoresed on a 2% agarose gel. GAPDH was used as a control housekeeping gene, as reported previously.

**Immunofluorescence double staining for AVP and Fos in the PVN and SON.** Immunohistochemical staining for AVP and cFos was performed 90 min after icv injection of 0.5 nmol NMS. Frozen brain sections were cut with a cryostat at a thickness of 18  $\mu$ m. The sections were pretreated with blocking solution comprising 1.5% donkey serum and 3% bovine serum albumin for 1 h, and then incubated for 2 days at 4 °C with rabbit anti-serum against rat AVP (Progen Biotechnik, Inc., Heidelberg, Germany) together with goat antiserum against rat cFos (Santa Cruz Biotechnology Inc. Cal., USA). After washing, the sections were incubated with a second antibody solution comprising Alexa-488-labeled anti-rabbit IgG antibody and Alexa-546-labeled donkey anti-goat IgG antibody solution (Molecular Probes, Inc.) for 30 min, followed by observation with a fluorescence microscope (Akisokope 2plus, Zeiss, Germany).

**Statistical analysis.** The data (means  $\pm$  SEM) were analyzed statistically by ANOVA and Student's *t* test. Differences at  $P < 0.05$  were considered statistically significant.

## Results and discussion

Plasma AVP levels after icv injection of NMS were significantly increased at a concentration of 0.2 and 2.0 nmol compared with the saline group (Fig. 1). The increase was observed at 5 min after icv injection, and continued for

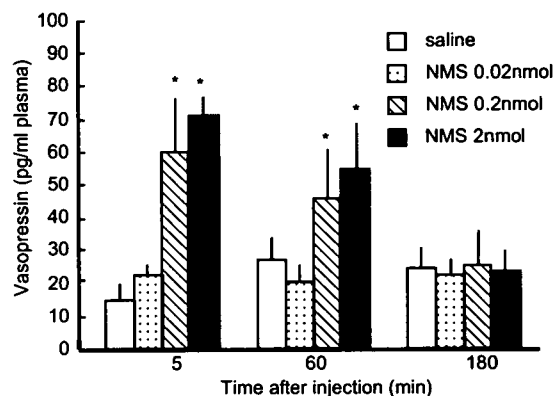


Fig. 1. Effect of icv administration of NMS on plasma AVP levels in rats. Whole blood was collected by decapitation at 5, 60, and 180 min after icv injection of 0.02, 0.2, and 2 nmol NMS or saline at 18:45 h. Each bar and vertical line represent means  $\pm$  SEM ( $n = 8$ ). Asterisks indicate significant differences from saline group ( $*P < 0.05$ ).

60 min. It has been shown that icv administration of neuromedin U increases the secretion of AVP, but that a very high dose (over 3 nmol) is required, and that the effect appears very slowly, with a peak at 15 and 30 min after administration [14]. Therefore, these results suggest that NMS rather than neuromedin U contributes to AVP secretion.

We then determined whether the NMS-induced increase of AVP plays a physiological role in urinary output. Icv administration of 0.2 and 2 nmol, but not 0.02 nmol, of NMS resulted in a significant decrease of urine volume during the dark period (Fig. 2A). This significant decrease did not continue until the following day (data not shown), and was unlikely to have been due to any side effect of NMS, since NMS-injected rats did not show any abnormal behavior (such as grooming, searching, attaching and barrel-rolling, which are caused by neuromedin U at doses exceeding 2 nmol). On the other hand, a larger dose of neuromedin U than that of NMS was required to cause a significant change in urinary volume, because neuromedin U at only 2 nmol caused a significant decrease (Fig. 2A). With regard to water intake, however, only a high dose of NMS caused a significant decrease during the dark period (Fig. 2B), and neuromedin U exerted no effect at any dose. These results indicate that NMS decreases urinary output during the

dark period in rats, independent of water intake. The fact that a small dose (0.2 nmol) of NMS, but not neuromedin U, affected urinary output suggests that NMS has a more predominant and physiological involvement in the regulation of urinary output than neuromedin U. Although it is unclear why the NMS-induced decrease in urinary output is more potent than that of neuromedin U, both peptides bind to same receptors with similar affinity *in vitro*. However, these differences between NMS and neuromedin U are also evident in terms of their anorexic effect and their phase-shifting effect on free-running rhythm [1,5,14]. The possibility that NMS acts on another unknown receptor cannot be excluded, and further studies will be required to investigate this issue.

To evaluate the participation of NMS in AVP secretion in detail, we examined the possibility of direct action of NMS on AVP neurons in the PVN and SON. RT-PCR analysis revealed that NMUR2 mRNA, but not NMUR1 mRNA, was expressed in both the PVN and SON in rats (Fig. 3A). In comparison with mRNA expression in spinal cord, NMUR2 mRNA expression in the PVN and SON was relatively weak. Expression of GAPDH mRNA was confirmed in all tissues (data not shown). In addition, cells immunostained with antiserum against NMUR2 were also observed in the PVN and SON (Fig. 3B1 and B2).

Immunohistochemical analysis revealed that AVP-containing neurons were distributed mainly in the magnocellular region of the PVN and a wide area of the SON (Fig. 3C1 and D1). In the PVN, cFos induced after icv injection of 0.2 nmol NMS was denser in the parvocellular region than in the magnocellular region (Fig. 3C2). Such cFos expression was partly observed in AVP neurons in the PVN and SON (Fig. 3C3 and D3). No effect was seen in saline-treated animals (data not shown).

We confirmed the presence of cFos expression in many neurons other than AVP neurons in the PVN and SON. In the PVN, the parvocellular region contains mainly corticotrophin releasing hormone (CRH) and AVP neurons, whereas the magnocellular region contains mainly OXT and AVP neurons [15,7]. In the SON, on the other hand, the dorsal and ventral magnocellular regions contain mainly OXT and AVP, respectively [7]. The wide expression of cFos after icv injection of NMS may indicate that NMS affects the secretion of not only AVP but also CRF and OXT. This possibility is supported by the fact that (1) NMUR2 is expressed on AVP- and OXT-containing neurons in the PVN [9], and (2) the NMS-induced anorexic effect was inhibited by a CRH antagonist, as reported previously [5].

Although the mechanism involved in the stimulation of AVP secretion by NMS is unknown, the following possibilities can be suggested. We have previously reported that the NMS mRNA expressions were observed in PVN and SON with relatively low levels [1], which may stimulate AVP secretion through a paracrine or autocrine action within these nuclei. We have also reported previously that NMS is dominantly expressed in the SCN and has a diurnal peak

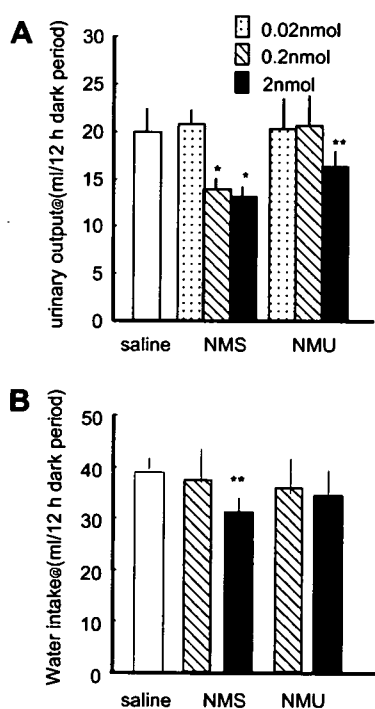


Fig. 2. Effect of icv administration of NMS on urinary output and water intake. Icv injections of saline as a control, or 0.02, 0.2, and 2 nmol of NMS and neuromedin U (NMU), were performed at 18:45 h and then urine volume (A) and water intake (B) were measured at 07:00 on the following morning. Each bar and vertical line represent means  $\pm$  SEM ( $n = 8$ ). Asterisks indicate significant differences from the saline group (\* $P < 0.01$ , \*\* $P < 0.05$ ).

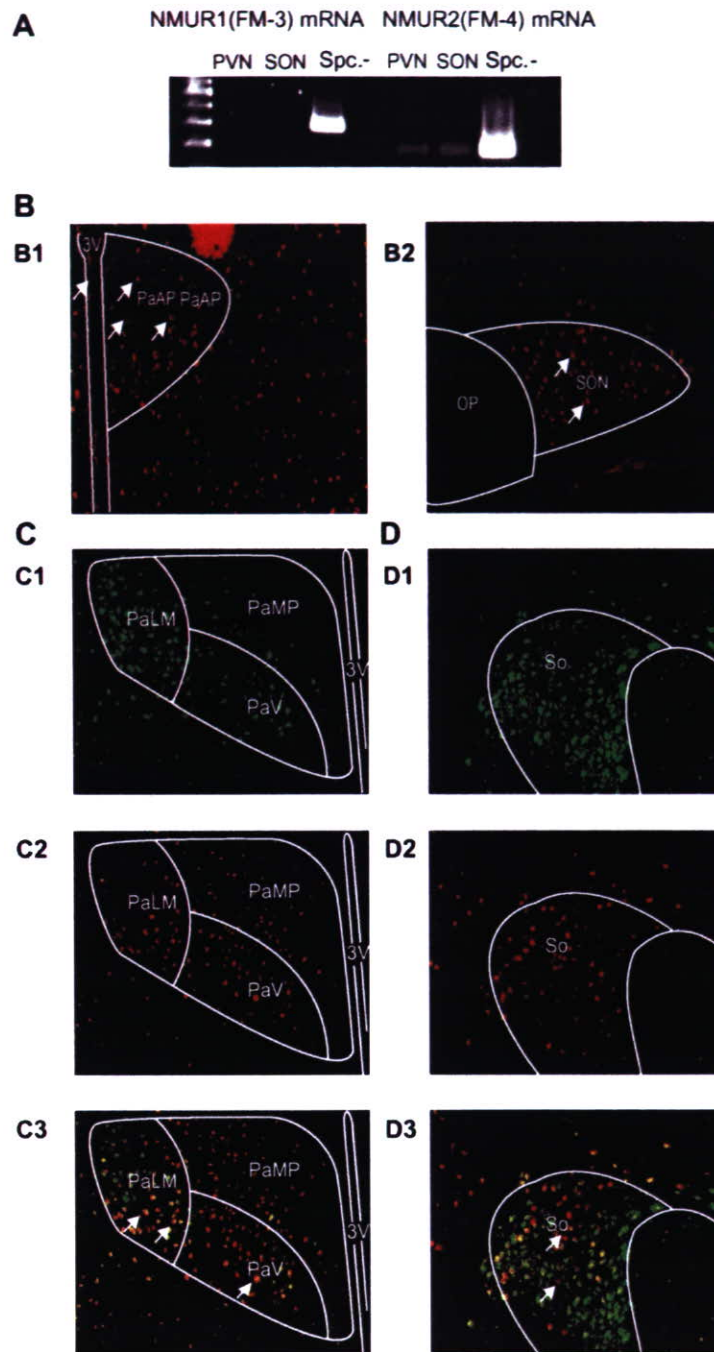


Fig. 3. (A) Expression of NMUR1 and NMUR2 mRNA in the PVN, SON, and spinal cord (Spc.). cDNA fragments from PVN and SON tissues punched out from frozen hypothalamic slices and spinal cord were amplified by PCR in the presence of oligonucleotide primers specific for NMUR1 and NMUR2. GAPDH mRNA expression was confirmed in RNA from all tissues (data not shown). (–) Indicates negative control. (B) Immunofluorescence staining for NMUR2 by anti-NMUR2 antiserum in a frozen hypothalamic slice. The area indicated by the white line in B1 and B2 shows the region of the PVN and SON, respectively. (C, D) Immunofluorescence staining for AVP in the PVN (C1) and SON (D1), and cFos expression after icv injection of 0.2 nmol NMS in the PVN (C2) and SON (D2). Dual immunostaining for cFos (red) and AVP (green) in the PVN (C3) and SON (D3). OP, optic chiasma; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaV, paraventricular hypothalamic nucleus, ventral part; PaMP, paraventricular hypothalamic nucleus, medial parvocellular part; PaAP, paraventricular hypothalamic nucleus, anterior parvocellular part; 3V, third ventricle. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

under light/dark conditions [1]. In rats, the plasma level of AVP also shows a circadian rhythm, being high in the light period and low in the dark period. Therefore, the circadian

rhythm of AVP may be reflected by NMS rhythm. If so, the rhythm of urinary output may be regulated by the rhythm of NMS in SCN. In which case, it may be specu-

lated that NMS secreted from SCN during diurnal period is transported to AVP neuron in SON and PVN through axonal projection, and shows diurnal antidiuretic action through stimulating the AVP secretion. During the nocturnal period, on the other hand, decrease of NMS from SCN may cause the decrease of AVP release and allow increasing of urinary output in rats.

In conclusion, the present findings indicate that central NMS may play an important role in urinary output through AVP secretion from the PVN and SON in rats.

#### Acknowledgments

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

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<sup>1</sup>Ghrelin Research Project, Translational Research Center, Kyoto University Hospital; <sup>2</sup>Department of Medicine and Clinical Science, Endocrinology and Metabolism, Kyoto University Graduate School of Medicine, Kyoto; and <sup>3</sup>Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

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

growth hormone secretagogue receptor; obesity; diabetes

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

Ghrelin is a 28-amino acid peptide with unique acylation modification, which is essential for its biological action (14). Ghrelin was originally identified in the rat stomach as an endogenous ligand for an orphan receptor, which so far has been called GH secretagogue receptor (GHS-R) (14). Ghrelin is involved in a wide variety of functions, including regulation of GH release, gastric acid secretion, gastric motility, blood pressure, and cardiac output (4, 8, 18, 19, 23, 28). Ghrelin also

has several metabolic functions, including orexigenic action (20, 22), reduction of insulin (5), and control of energy expenditure (24), which are all involved in the pathophysiology of adiposity or diabetes.

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

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

### MATERIALS AND METHODS

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

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

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

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

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

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

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

**Measurements of plasma ghrelin concentrations.** Measurement of plasma ghrelin levels was performed as reported previously (12). Briefly, blood was drawn from the retroorbital vein after an overnight fast and then immediately transferred to chilled siliconized glass tubes containing Na<sub>2</sub>EDTA (1 mg/ml) and aprotinin (1,000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan) and centrifuged at 4°C. Immediately after the plasma was separated, hydrochloric acid was added to samples at final concentration of 0.1 N. Plasma was immediately frozen and stored at -80°C until assay. Plasma ghrelin concentrations were determined using an active ghrelin ELISA kit that recognizes *n*-octanoylated ghrelin (Mitsubishi Kagaku Iatron, Tokyo, Japan) (1).

**Real-time quantitative RT-PCR.** Total RNA was extracted from the pituitary and hypothalamus using a Sepasol RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription (RT) was performed in the presence of random hexamers with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), using the following primers and TaqMan probes: mouse GH sense, 5'-AAGAGTTCGAGCGTG-CCTACA-3', and antisense, 5'-GAAGCAATCCATGTCGGTTC-3', with the TaqMan probe, 5'-CCATTCAGAATGCCAGGCTGCTTTC-3'; mouse GHRH receptor (GHRH-R) sense, 5'-GCCCTTGAACGTGTA-ACCA-3', and antisense, 5'-GCAACCAGGATGGCAATAGC-3', with the TaqMan probe, 5'-AGCATCTCCATTGTAGCCCTCTGCGTG-3'; mouse GHS-R sense, 5'-CACCAACCTCTACCTATCCAGCAT-3', and antisense, 5'-CTGACAACTGGAAGAGTTTGCA-3', with the TaqMan probe, 5'-TCCGATCTGCTCATCTCTCTGTGCATG-3'; mouse ghrelin sense, 5'-GCATGCTCTGGATGGACATG-3', and antisense, 5'-TGGTG-GCTTCTTGATTCCT-3', with the TaqMan probe, 5'-AGCCAGAG-CACCAGAAAGCCCA-3'; mouse somatostatin receptor (SSTR)2 sense, 5'-GGTCAAGGCAGACAATTCACAA -3', and antisense, 5'-GTGT-TAGCACACATACACACAGGACTT -3', with the TaqMan probe, 5'-CGGCAGAAACCGGAAAAACCAAACTAAAT -3'; mouse SSTR5 sense, 5'-CGCTGCCTGACCGCTAAGTA-3', and antisense, 5'-GCTCACAGAGGTTGGCTCACA-3', with the TaqMan probe, 5'-CTGCACAGGAGAGGTCTCCACGGCT-3'; mouse GHRH sense, 5'-AGGATGCAGCGACACGTAGA-3', and antisense, 5'-TCTCCCCTT-GCTTGTTCATGA-3', with the TaqMan probe, 5'-CCACCAACTACAG-GAAACTCCTGAGCCA-3'. The mRNA expression in each gene was normalized to that of 18s ribosomal RNA.

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

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

## RESULTS

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

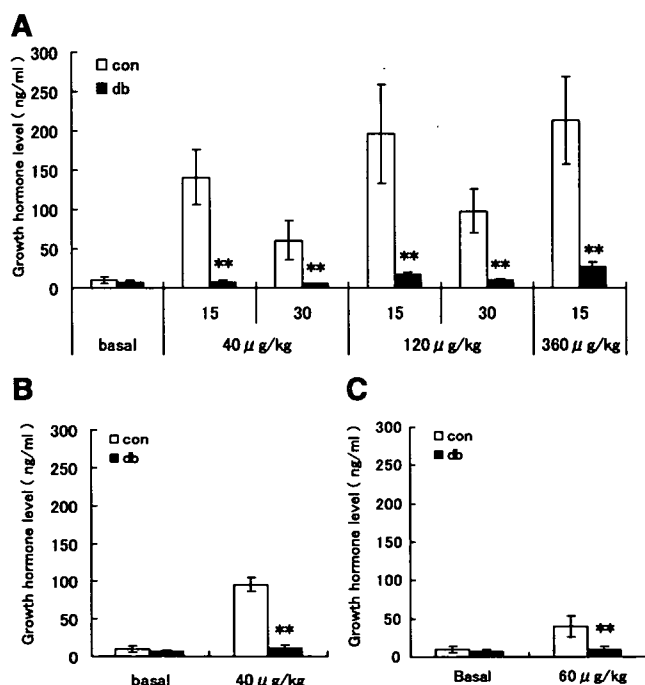


Fig. 1. Growth hormone (GH) responses to ghrelin in *db/db* mice. A: serum GH levels 15 or 30 min after sc injection of ghrelin into *db/db* (db) or control (con) mice. B: serum GH levels 15 min after iv ghrelin injection into db or con mice. C: serum GH levels 15 min after sc injection of GH-releasing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

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

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

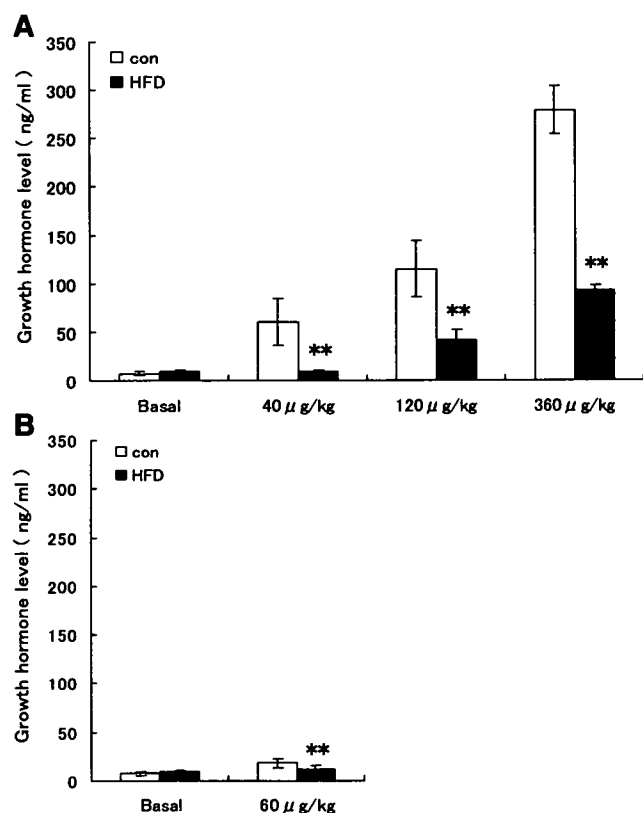


Fig. 2. GH responses to ghrelin in a diet-induced obesity mouse model. A: serum GH levels 15 min after sc injection of ghrelin into mice on a high-fat diet (HFD) or con mice. B: serum GH levels 15 min after GHRH sc injection. \*\* $P < 0.01$  compared with controls;  $n = 7$ .

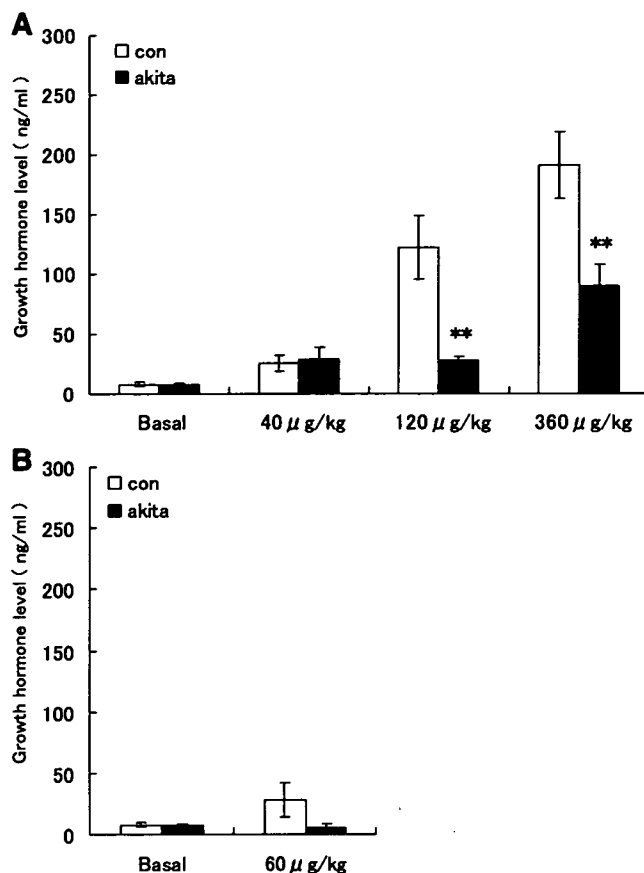


Fig. 3. GH responses to ghrelin in Akita mice. A: serum GH levels 15 min after sc injection of ghrelin into Akita or con mice. B: serum GH levels 15 min after sc injection of GHRH. \*\* $P < 0.01$  compared with control;  $n = 7$ .

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

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

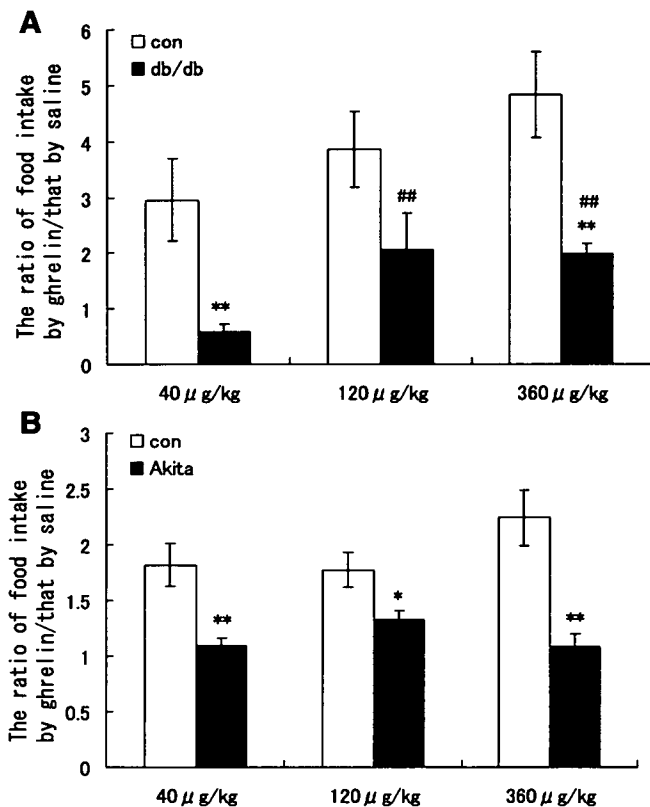


Fig. 4. Food intake stimulated by sc ghrelin injection. A: the ratio of 1-h food intake after sc ghrelin injection to that observed after sc saline injection in db and con mice. B: the ratio of the food intake observed for 1 h after sc ghrelin injection to that seen after saline injection for Akita and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

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

Finally, we examined the effect of chronic ghrelin injection to HFD mice. To maximize GH-stimulating activity of ghrelin and to minimize orexigenic action of ghrelin, we first examined GH responses to low-dose ghrelin with GHRH coadministration in HFD mice. Acute GH responses to ghrelin were significantly potentiated by coadministration of GHRH even at the lowest dose in HFD mice (Fig. 6A). By 10 days of twice daily injections of saline or ghrelin and GHRH, both control and HFD mice lose weight by ~6–8%. This weight reduction might be due to stress of twice daily injection, which is usually

covered by growth in younger mice. Control mice treated with ghrelin and GHRH tended to take in more food than those with saline (Fig. 6C). Fat masses were more preserved in the ghrelin- and GHRH-treated groups than in the saline-treated group in control mice (Fig. 6, B, D, and E), although percent body weight and percent lean body mass changes were comparable between the saline-treated and the ghrelin- and GHRH-treated group in control mice. In HFD mice, food intake, percent body weight change, and percent lean body mass change were comparable between the saline-treated group and the ghrelin- and GHRH-treated groups (Fig. 6, B, D, and E). In contrast to control mice, fat mass tended to even be decreased in the ghrelin and GHRH group than in the saline-treated group in HFD mice (Fig. 6D). In both control and HFD mice, blood glucose, serum insulin, and serum IGF-I levels of the ghrelin- and GHRH-treated group were not significantly different from those of the saline-treated group (Fig. 6, F, G, and H).

## DISCUSSION

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

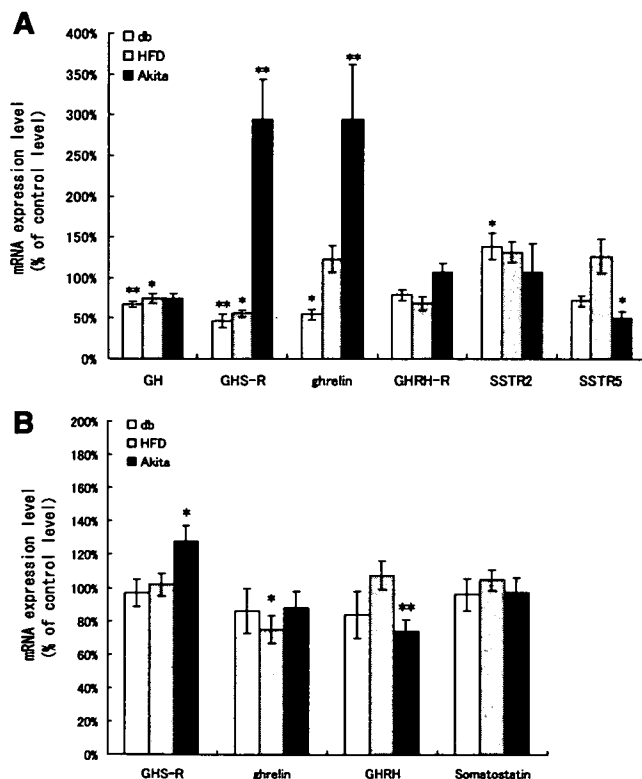


Fig. 5. The mRNA expression levels in the pituitaries or hypothalamuses of db mice, mice on a HFD, and Akita mice. A: the mRNA expression levels of GH, GH secretagogue receptor (GHS-R), ghrelin, GH-releasing hormone receptor (GHRH-R), somatostatin receptor (SSTR)2, and SSTR5 in the pituitaries of db,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

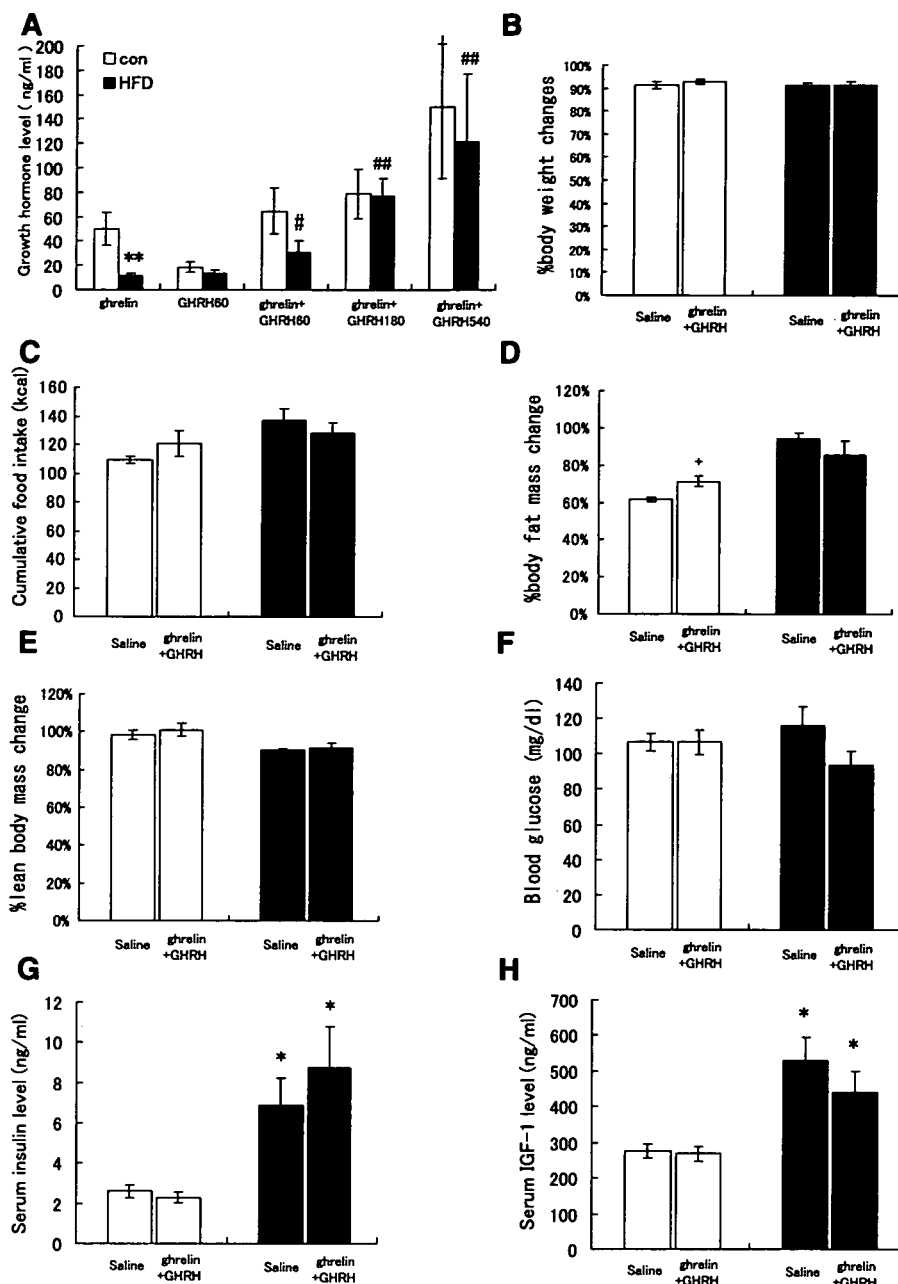


Fig. 6. Chronic treatment of ghrelin and GHRH on mice on a HFD. A: serum GH levels 15 or 30 min after sc injection of 40  $\mu$ g/kg ghrelin or 60  $\mu$ g/kg GHRH or ghrelin and GHRH (60, 180, 540  $\mu$ g/kg) into HFD or con mice. B: %Body weight, fat mass (D), and lean body mass (E) changes before and after treatment of 40  $\mu$ g/kg ghrelin and 60  $\mu$ g/kg GHRH or saline for 10 days in control mice (open bars) or mice on a HFD (filled bars). C: cumulative food intake for 10 days. Blood glucose (F), serum insulin level (G), and serum IGF-1 level (H) after 10 days administration of ghrelin and GHRH. \* $P$  < 0.05; \*\* $P$  < 0.01 compared with control; # $P$  < 0.05; ## $P$  < 0.01 compared with ghrelin; + $P$  < 0.05 compared with saline;  $n$  = 7.

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

which verify the use of *db/db* and HFD mice as experimental animal models for ghrelin treatment for obesity.

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

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

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

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

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

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

In conclusion, we demonstrated that acute GH responses to ghrelin were suppressed in both genetic and diet-induced mouse models of obesity. The decreased pituitary levels of GHS-R mRNA may contribute to suppression of GH response. We also demonstrated that acute GH responses to ghrelin were suppressed in Akita mice, an insulin-deprived diabetic mouse model. Decreased GHRH mRNA levels in hypothalamus and the lack of stimulation of food intake by ghrelin indicate the involvement of hypothalamus in the mechanism of suppressed GH response to ghrelin in Akita mice. These results indicate that suppressed GH response to ghrelin has a different mechanism in obese and insulin-resistant mice and insulin-deprived diabetic animals. In addition, HFD mice injected with ghrelin and GHRH showed potentiated GH responses. Chronic treat-

ment of low-dose ghrelin and GHRH did not promote fat deposition in HFD mice. These results indicate that low-dose ghrelin and GHRH administration at least does not worsen obesity and that it may restore suppressed GH secretion.

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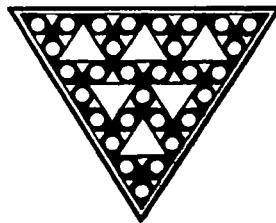
#### GRANTS

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## Ghrelin stimulates growth hormone secretion and food intake in aged rats

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### Abstract

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

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**Keywords:** Ghrelin; Feeding behavior; Growth hormone secretion; Ageing

### 1. Introduction

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

Ghrelin, a 28-amino-acid peptide, was isolated from human and rat stomachs as an endogenous ligand of growth hormone

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

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

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

## 2. Materials and methods

### 2.1. Animals

Male Long-Evans Tokushima Otsuka (LETO) rats (4-week-old) were obtained from Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All animals were housed individually in plastic cages at constant room temperature (23 °C) in a 12 h light (8 AM–8 PM)/12 h dark cycle and were given standard laboratory chow and water *ad libitum*. Experiments were conducted on rats at 3, 12, 24, and 27 months of age ( $n = 10$ ). In this study, anesthesia was carried out by an intraperitoneal (ip) injection of sodium pentobarbital (75 mg/kg body weight) (Abbot Lab., Chicago, IL). Rats were used as follows. Sterilized intravenous (iv) cannulae were implanted into the right jugular vein 1 week before the experiments of feeding and GH response on rats at 3, 12, 24, and 27 months of age. All rats recovered from surgery within 1 week, showing food intake amounts similar to pre-surgery levels and progressive weight gain. These rats were then used in the experiments. Rat ghrelin (Peptide Institute Inc., Osaka, Japan) or saline was administered icv to rats fed *ad libitum*. The 2 h food intake amounts were then measured. This feeding test

was performed using a crossover design experiment in which animals were randomized to receive either test substance with a washout period of 2 days between each administration. Two days after the feeding test, ghrelin (10 nmol/kg body weight) was administered iv to these rats which were anesthetized by sodium pentobarbital for the GH response test. After these tests, the iv cannulae were removed from the rats using sterilized devices. To prevent suppuration by infection, we frequently disinfected the rat, and exchanged cages after the operation. Rats were bred in previously described conditions until reaching the age of the following test. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care.

### 2.2. Food intake

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

### 2.3. GH response

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

### 2.4. Statistic analysis

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

## 3. Results

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

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

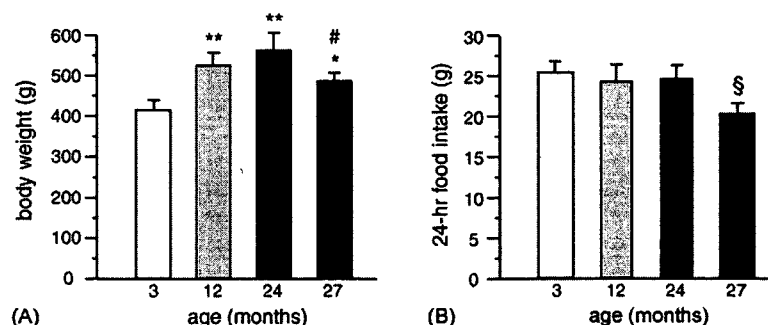


Fig. 1. Changes of body weight (A) and 24 h food intake (B) with ageing. \**P* < 0.01, \*\**P* < 0.001 vs. 3-month-old rats, #*P* < 0.05 vs. 24-month-old, \$*P* < 0.01 vs. 3-, 12-, or 24-month-old rats.

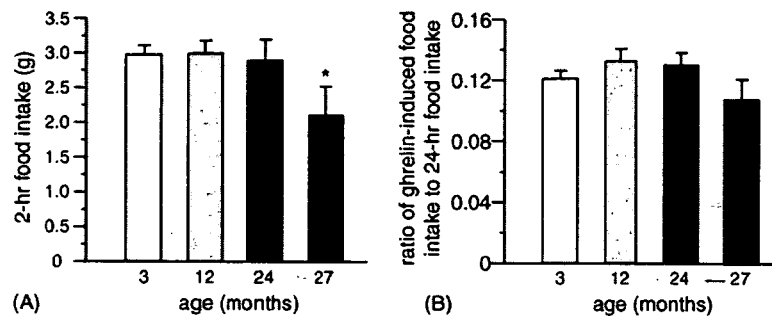


Fig. 2. (A) Effect of iv administration of ghrelin (10 nmol/kg body weight) on 2 h food intake in 3-, 12-, 24- and 27-month-old rats. \* $P < 0.01$  vs. 3-, 12-, or 24-month-old rats. (B) No effect of ageing on the ratio of ghrelin-induced 2 h food intake to 24 h food intake.

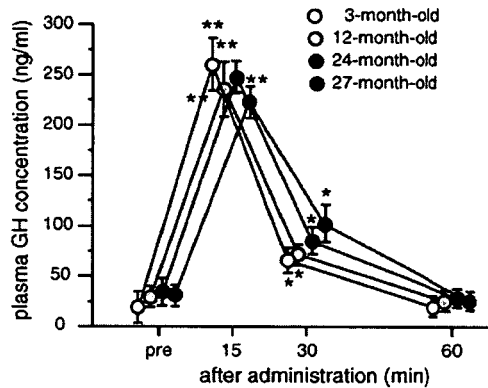


Fig. 3. Effect of iv administration of ghrelin (10 nmol/kg body weight) on the plasma GH concentration in 3-, 12-, 24- and 27-month-old rats. \* $P < 0.01$ , \*\* $P < 0.001$  vs. pre-administration.

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

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

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

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

## 4. Discussion

The circulation level of insulin-like growth factor-1 (IGF-1) is increased by the increase in plasma GH concentration. GH and IGF-1 promote cell survival and proliferation through

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

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

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

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

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

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