Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 361 (2007) 457–461
BBRC
www.elsevier.com/locate/ybbrc

Neuromedin S exerts an antidiuretic action in rats

Takumi Sakamoto [a], Kenji Mori [b], Keiko Nakahara [a], Mikiya Miyazato [b], Kenji Kangawa [b], Hiroshi Sameshima [c], Noboru Murakami [a,*]

[a] Department of Veterinary Physiology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan
[b] Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan
[c] Department of Obstetrics and Gynecology, Faculty of Medicine, Miyazaki University, Miyazaki 889-2192, Japan

Received 4 July 2007
Available online 18 July 2007

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 supachiasmatic 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.

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 NMU1R and NMU2R 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

* Corresponding author. Fax: +81 985 58 7265.
E-mail address: a0d201u@cc.miyazaki-u.ac.jp (N. Murakami).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.07.036

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, NMU2R 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 ± 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 μl of the solution was injected into each free-moving rat through a 27-gauge injection cannula connected to a 50-μl 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-μm-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 μg 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 NMUR1and NMUR2 were as follows: rat NMUR1 primer set: 5′-C ACGACTCCCATAGCCA-3′ (sense), 5′-TCACACCCTGGATCCCT 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 μm. 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 antiserum 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 (Akisoskope 2plus, Zeiss, Germany).

Statistical analysis. The data (means ± 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

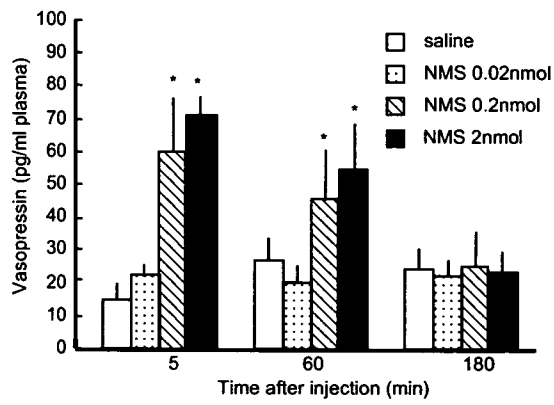

Fig. 1. Effect of icv administration of NMS on plasma AVP levels in rats. Whole blood was collected by decapitation at 5, 60, and 180 min after icv injection of 0.02, 0.2, and 2 nmol NMS or saline at 18:45 h. Each bar and vertical line represent means ± SEM ($n = 8$). Asterisks indicate significant differences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

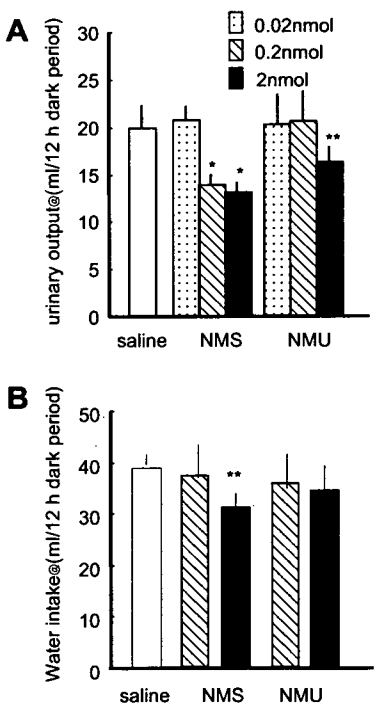

Fig. 2. Effect of icv administration of NMS on urinary output and water intake. Icv injections of saline as a control, or 0.02, 0.2, and 2 nmol of NMS and neuromedin U (NMU), were performed at 18:45 h and then urine volume (A) and water intake (B) were measured at 07:00 on the following morning. Each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

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

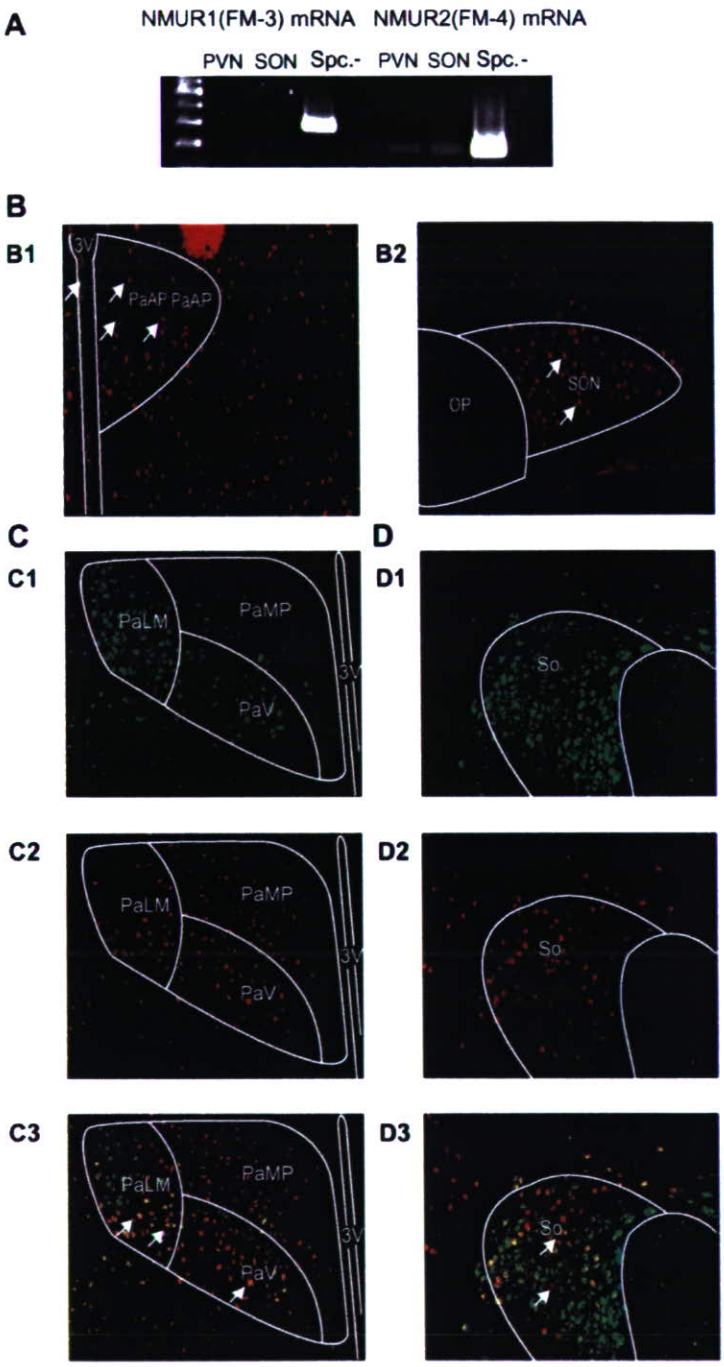

Fig. 3. (A) Expression of NMUR1 and NMUR2 mRNA in the PVN, SON, and spinal cord (Spc.). cDNA fragments from PVN and SON tissues punched out from frozen hypothalamic slices and spinal cord were amplified by PCR in the presence of oligonucleotide primers specific for NMUR1 and NMUR2. GAPDH mRNA expression 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 parvicellular 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

We thank Y. Mori for technical assistance. This study was supported in part by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN), Grant-in-Aid for Scientific Research(s) from the Ministry of Education, Science, Sports, and Culture of Japan (N.M), and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (K.K).

References

[1] K. Mori, M. Miyazato, T. Ida, N. Murakami, R. Serino, Y. Ueta, M. Kojima, K. Kangawa, Identification of neuromedin S and its possible role in the mammalian circadian oscillator system, EMBO J. 24 (2005) 325–335.

[2] A.D. Howard, R. Wang, S.S. Pong, T.N. Mellin, A. Strack, X.M. Guan, Z. Zeng, D.L. Williams, S.D. Feighner, C.N. Nunes, B. Murphy, J.N. Stair, H. Yu, Q. Jiang, M.K. Clements, C.P. Tan, K.K. McKee, D.L. Hreniuk, T.P. McDonald, K.R. Lynch, J.F. Evans, C.P. Austin, C.T. Caskey, L.H. Van der Ploeg, Q. Liu, Identification of receptors for neuromedin U and its role in feeding, Nature 406 (2000) 70–74.

[3] R. Fuji, M. Hosoya, S. Fukusumi, Y. Kawamata, Y. Habatae, S. Hinuma, H. Onda, O. Nishimura, M. Fujino, Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3, J. Biol. Chem. 275 (2000) 21068–21074.

[4] M. Kojima, R. Haruno, M. Nakazato, Y. Date, N. Murakami, R. Hanada, H. Matsuo, K. Kangawa, Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3), Biochem. Biophys. Res. Commun. 276 (2000) 435–438.

[5] T. Ida, K. Mori, M. Miyazato, Y. Egi, S. Abe, K. Nakahara, M. Nishihara, K. Kanagawa, N. Murakami, Neuromedin S is a novel anorexigenic hormone, Endocrinology 146 (2005) 4217–4223.

[6] E. Vigo, J. Roa, M. López, J.M. Castellano, R. Fernandez-Fernandez, V.M. Navarro, R. Pineda, E. Aguilar, C. Diéguez, L. Pinilla, M. Tena-Sempere, Neuromedin S as novel putative regulator of luteinizing hormone secretion, Endocrinology 148 (2007) 813–823.

[7] C.H. Rhodes, J.I. Morrell, D.W. Pfaff, Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin, J. Comp. Neurol. 198 (1981) 45–64.

[8] L.W. Swanson, P.E. Sawchenko, Hypothalamic integration: organization of the paraventricular and supraoptic nuclei, Annu. Rev. Neurosci. 6 (1983) 269–324.

[9] D.L. Qiu, C.P. Chu, H. Tsukino, T. Shirasaka, H. Nakao, K. Kato, T. Kunitake, T. Katoh, H. Kannan, Neuromedin U receptor-2 mRNA and HCN channels mRNA expression in NMU-sensitive neurons in rat hypothalamic paraventricular nucleus, Neurosci. Lett. 374 (2005) 69–72.

[10] K. Nakahara, M. Nakagawa, Y. Baba, M. Sato, K. Toshinai, Y. Date, M. Nakazato, M. Kojima, M. Miyazato, H. Kaiya, H. Hosoda, K. Kangawa, N. Murakami, Maternal ghrelin plays an important role in fetal development during pregnancy, Endocrinology 147 (2006) 1333–1342.

[11] N. Murakami, K. Takahashi, Circadian rhythm of adenosine-3',5'-monophosphate content in suprachiasmatic nucleus (SCN) and ventromedial hypothalamus (VMH) in the rat, Brain Res. 276 (1983) 297–304.

[12] H. Zeng, A. Gragerov, J.G. Hohmann, M.N. Pavlova, B.A. Schimpf, H. Xu, L.J. Wu, H. Toyoda, M.G. Zhao, A.D. Rohde, G. Gragerova, R. Onrust, J.E. Bergmann, M. Zhuo, G.A. Gaitanaris, Neuromedin U receptor 2-deficient mice display differential responses in sensory perception, stress, and feeding, Mol. Cell Biol. 26 (2006) 9352–9363.

[13] K. Nakahara, R. Hanada, N. Murakami, H. Teranishi, H. Ohgusu, N. Fukushima, M. Moriyama, T. Ida, K. Kangawa, M. Kojima, The gut-brain peptide neuromedin U is involved in the mammalian circadian oscillator system, Biochem. Biophys. Res. Commun. 318 (2004) 156–161.

[14] Y. Ozaki, T. Onaka, M. Nakazato, J. Saito, K. Kanemoto, T. Matsumoto, Y. Ueta, Centrally administered neuromedin U activates neurosecretion and induction of c-fos messenger ribonucleic acid in the paraventricular and supraoptic nuclei of rat, Endocrinology 143 (2002) 4320–4329.

[15] P.E. Sawchenko, L.W. Swanson, Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain, Fed. Proc. 44 (1985) 221–227.

Am J Physiol Endocrinol Metab 293: E819–E825, 2007.
First published June 26, 2007; doi:10.1152/ajpendo.00681.2006.

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

Hiroshi Iwakura,[1] Takashi Akamizu,[1] Hiroyuki Ariyasu,[1] Taiga Irako,[1] Kiminori Hosoda,[2] Kazuwa Nakao,[2] and Kenji Kangawa[1,3]

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

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

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

growth hormone secretagogue receptor; obesity; diabetes

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

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

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

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

MATERIALS AND METHODS

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

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

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

Address for reprint requests and other correspondence: H. Iwakura, Ghrelin Research Project, Translational Research Center, Kyoto University Hospital, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (e-mail: hiwaku@kuhp.kyoto-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

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

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

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

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

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

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

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

RESULTS

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

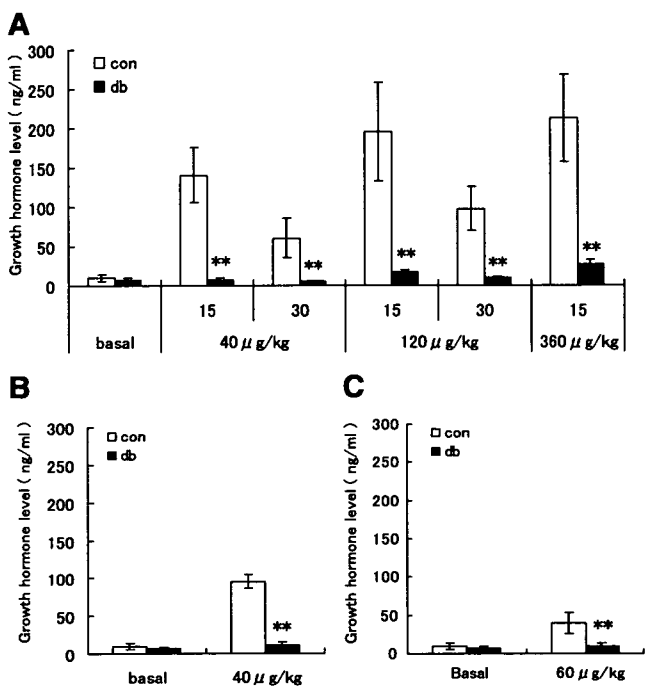

Fig. 1. Growth hormone (GH) responses to ghrelin in *db/db* mice. *A*: serum GH levels 15 or 30 min after sc injection of ghrelin into *db/db* (db) or control (con) mice. *B*: serum GH levels 15 min after iv ghrelin injection into db or con mice. *C*: serum GH levels 15 min after sc injection of GH-releasing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

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

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

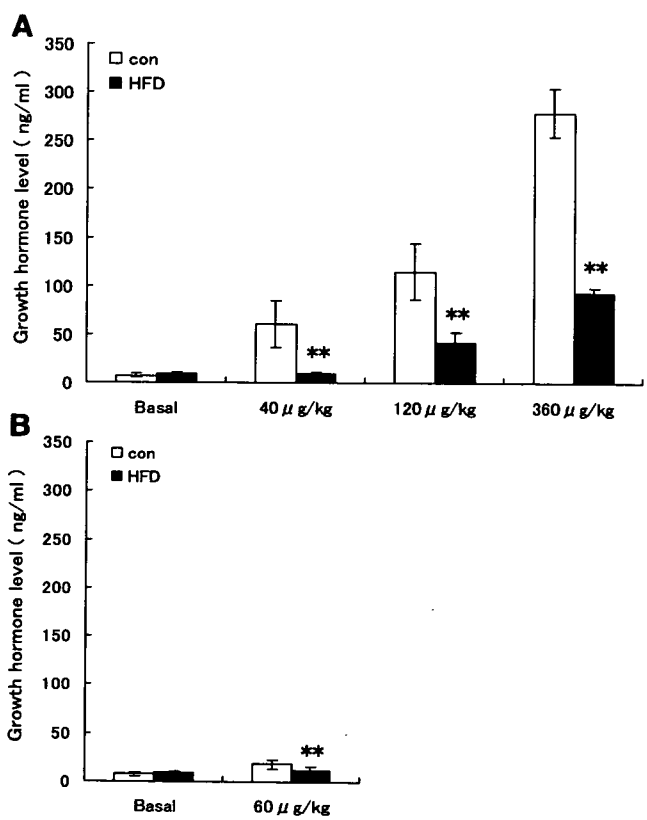

Fig. 2. GH responses to ghrelin in a diet-induced obesity mouse model. *A*: serum GH levels 15 min after sc injection of ghrelin into mice on a high-fat diet (HFD) or con mice. *B*: serum GH levels 15 min after GHRH sc injection. $^{**}P < 0.01$ compared with controls; $n = 7$.

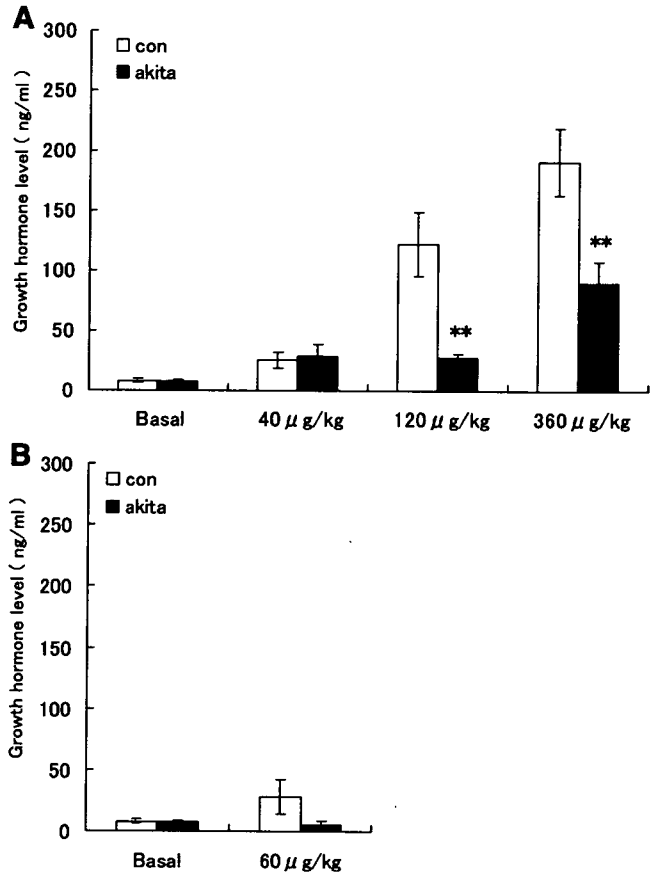

Fig. 3. GH responses to ghrelin in Akita mice. *A*: serum GH levels 15 min after sc injection of ghrelin into Akita or con mice. *B*: serum GH levels 15 min after sc injection of GHRH. $^{**}P < 0.01$ compared with control; $n = 7$.

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

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

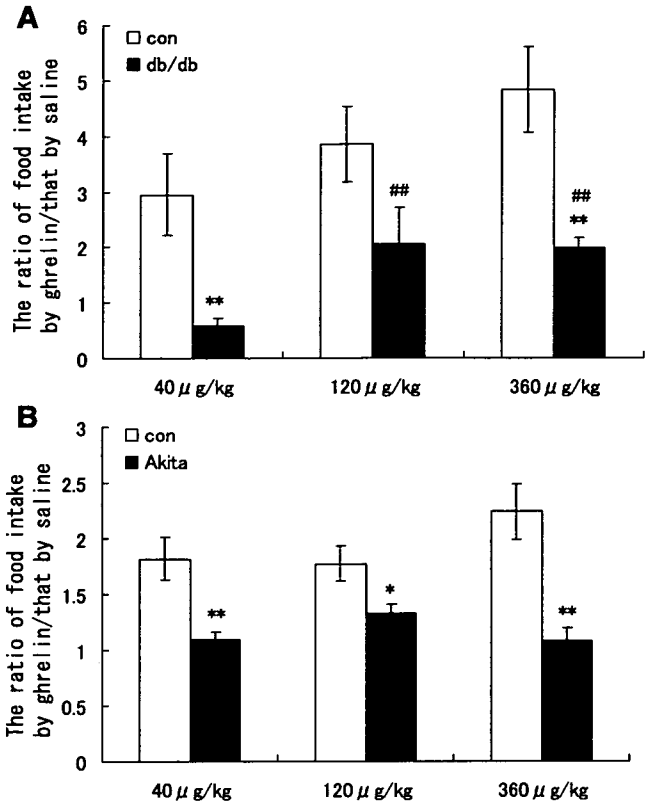

Fig. 4. Food intake stimulated by sc ghrelin injection. *A*: the ratio of 1-h food intake after sc ghrelin injection to that observed after sc saline injection in db and con mice. *B*: the ratio of the food intake observed for 1 h after sc ghrelin injection to that seen after saline injection for Akita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

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

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

DISCUSSION

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

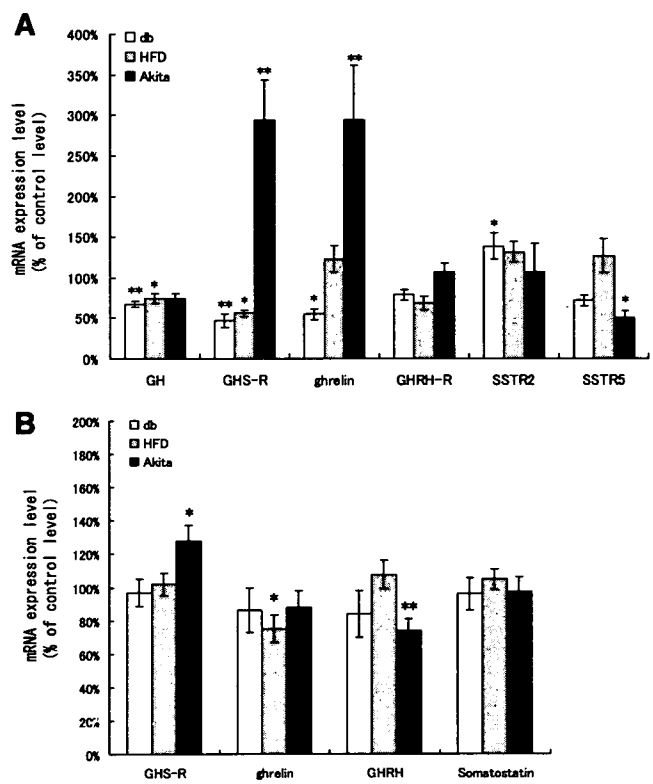

Fig. 5. The mRNA expression levels in the pituitaries or hypothalamuses of db mice, mice on a HFD, and Akita mice. *A*: the mRNA expression levels of GH, GH secretagogue receptor (GHS-R), ghrelin, GH-releasing hormone receptor (GHRH-R), somatostatin receptor (SSTR)2, and SSTR5 in the pituitaries of db,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

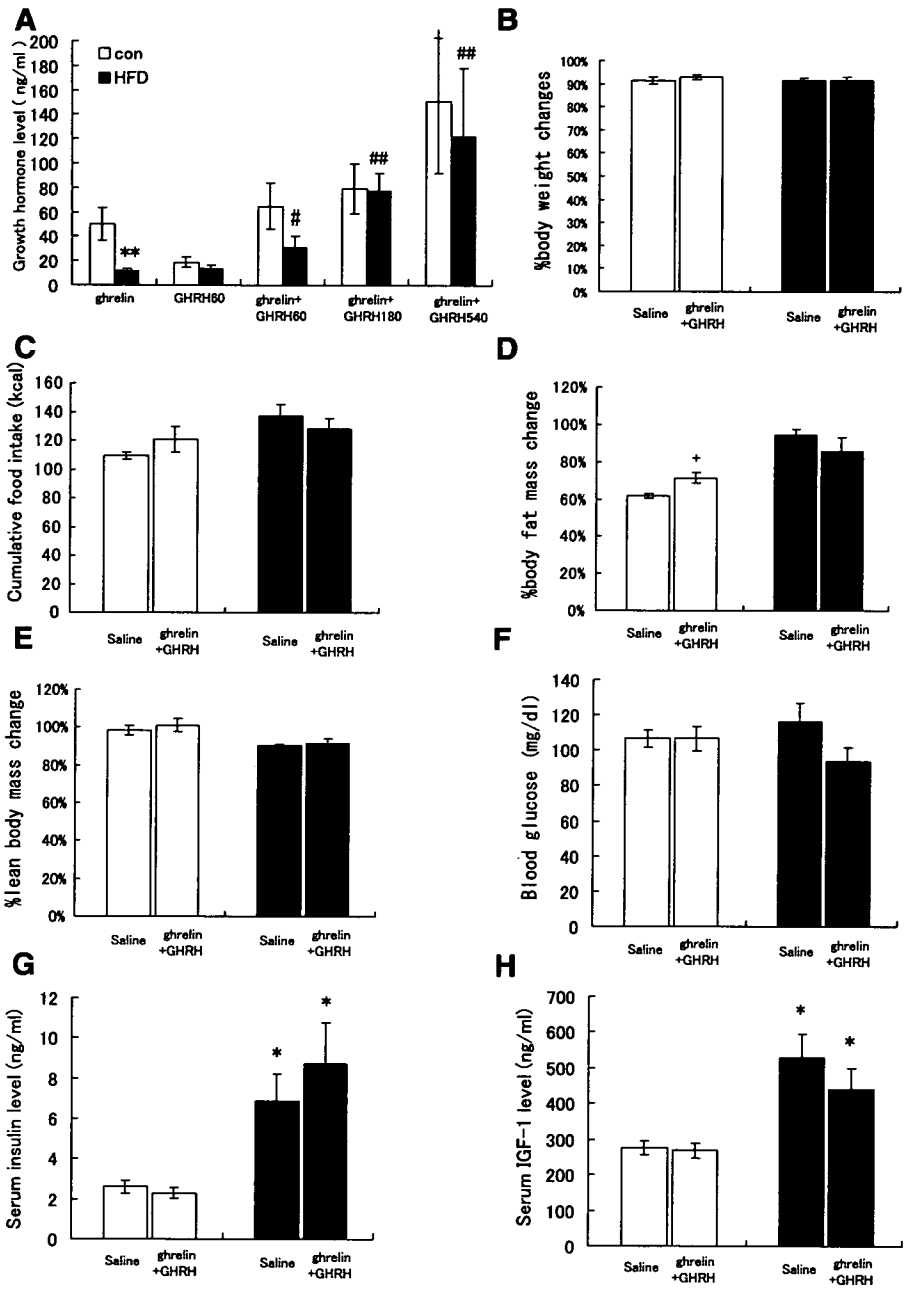

Fig. 6. Chronic treatment of ghrelin and GHRH on mice on a HFD. *A*: serum GH levels 15 or 30 min after sc injection of 40 μg/kg ghrelin or 60 μg/kg GHRH or ghrelin and GHRH (60, 180, 540 μg/kg) into HFD or con mice. %Body weight (*B*), fat mass (*D*), and lean body mass (*E*) changes before and after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

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

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

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

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

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

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

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

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

ACKNOWLEDGMENTS

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

GRANTS

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

REFERENCES

1. **Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Kangawa K.** Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *J Clin Endocrinol Metab* 90: 6–9, 2005.
2. **Alvarez-Castro P, Isidro ML, Garcia-Buela J, Leal-Cerro A, Broglio F, Tassone F, Ghigo E, Dieguez C, Casanueva FF, Cordido F.** Marked GH secretion after ghrelin alone or combined with GH-releasing hormone (GHRH) in obese patients. *Clin Endocrinol (Oxf)* 61: 250–255, 2004.
3. **Argente J, Caballo N, Barrios V, Pozo J, Munoz MT, Chowen JA, Hernandez M.** Multiple endocrine abnormalities of the growth hormone and insulin-like growth factor axis in prepubertal children with exogenous obesity: effect of short- and long-term weight reduction. *J Clin Endocrinol Metab* 82: 2076–2083, 1997.
4. **Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E.** Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J Endocrinol Invest* 23: 493–495, 2000.
5. **Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E.** Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J Clin Endocrinol Metab* 86: 5083–5086, 2001.
6. **Cordido F, Fernandez T, Martinez T, Penalva A, Peino R, Casanueva FF, Dieguez C.** Effect of acute pharmacological reduction of plasma free fatty acids on growth hormone (GH) releasing hormone-induced GH secretion in obese adults with and without hypopituitarism. *J Clin Endocrinol Metab* 83: 4350–4354, 1998.
7. **Cutfield WS, Wilton P, Bennmarker H, Albertsson-Wikland K, Chatelain P, Ranke MB, Price DA.** Incidence of diabetes mellitus and impaired glucose tolerance in children and adolescents receiving growth-hormone treatment. *Lancet* 355: 610–613, 2000.
8. **Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, Nakazato M.** Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. *Biochem Biophys Res Commun* 275: 477–480, 2000.
9. **Fintini D, Alba M, Schally AV, Bowers CY, Parlow AF, Salvatori R.** Effects of combined long-term treatment with a growth hormone-releasing hormone analogue and a growth hormone secretagogue in the growth hormone-releasing hormone knock out mouse. *Neuroendocrinology* 82: 198–207, 2005.
10. **Furuhata Y, Kagaya R, Hirabayashi K, Ikeda A, Chang KT, Nishihara M, Takahashi M.** Development of obesity in transgenic rats with low circulating growth hormone levels: involvement of leptin resistance. *Eur J Endocrinol* 143: 535–541, 2000.
11. **Giustina A, Veldhuis JD.** Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. *Endocr Rev* 19: 717–797, 1998.
12. **Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K.** Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. *J Biol Chem* 280: 15247–15256, 2005.
13. **Kamegai J, Tamura H, Shimizu T, Ishii S, Tatsuguchi A, Sugihara H, Oikawa S, Kineman RD.** The role of pituitary ghrelin in growth hormone (GH) secretion: GH-releasing hormone-dependent regulation of pituitary

ghrelin gene expression and peptide content. *Endocrinology* 145: 3731–3738, 2004.

14. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K.** Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660, 1999.
15. **Luque RM, Kineman RD.** Impact of obesity on the growth hormone (GH)-axis: evidence for a direct inhibitory effect of hyperinsulinemia on pituitary function. *Endocrinology* 147: 2754–2763, 2006.
16. **Maccario M, Tassone F, Gianotti L, Lanfranco F, Grottoli S, Arvat E, Muller EE, Ghigo E.** Effects of recombinant human insulin-like growth factor I administration on the growth hormone (gh) response to GH-releasing hormone in obesity. *J Clin Endocrinol Metab* 86: 167–171, 2001.
17. **Mano-Otagiri A, Nemoto T, Sekino A, Yamauchi N, Shuto Y, Sugihara H, Oikawa S, Shibasaki T.** Growth hormone-releasing hormone (GHRH) neurons in the arcuate nucleus (Arc) of the hypothalamus are decreased in transgenic rats whose expression of ghrelin receptor is attenuated: evidence that ghrelin receptor is involved in the up-regulation of GHRH expression in the arc. *Endocrinology* 147: 4093–4103, 2006.
18. **Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K.** Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* 276: 905–908, 2000.
19. **Nagaya N, Uematsu M, Kojima M, Ikeda Y, Yoshihara F, Shimizu W, Hosoda H, Hirota Y, Ishida H, Mori H, Kangawa K.** Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. *Circulation* 104: 1430–1435, 2001.
20. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S.** A role for ghrelin in the central regulation of feeding. *Nature* 409: 194–198, 2001.
21. **Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA, Ukkola O.** Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* 52: 2546–2553, 2003.
22. **Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K.** Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 50: 227–232, 2001.
23. **Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K.** Ghrelin strongly stimulates growth hormone release in humans. *J Clin Endocrinol Metab* 85: 4908–4911, 2000.
24. **Tschop M, Smiley DL, Heiman ML.** Ghrelin induces adiposity in rodents. *Nature* 407: 908–913, 2000.
25. **Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML.** Circulating ghrelin levels are decreased in human obesity. *Diabetes* 50: 707–709, 2001.
26. **Veldhuis JD, Iranmanesh A, Ho KK, Waters MJ, Johnson ML, Lizarralde G.** Dual defects in pulsatile growth hormone secretion and clearance subserve the hyposomatotropism of obesity in man. *J Clin Endocrinol Metab* 72: 51–59, 1991.
27. **Wren AM, Small CJ, Fribbens CV, Neary NM, Ward HL, Seal LJ, Ghatei MA, Bloom SR.** The hypothalamic mechanisms of the hypophysiotropic action of ghrelin. *Neuroendocrinology* 76: 316–324, 2002.
28. **Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR.** The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141: 4325–4328, 2000.
29. **Yoshioka M, Kayo T, Ikeda T, Koizumi A.** A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 46: 887–894, 1997.

Available online at www.sciencedirect.com
ScienceDirect
Mechanisms of Ageing and Development 128 (2007) 182–186
Mechanisms of Ageing and Development
www.elsevier.com/locate/mechagedev

Ghrelin stimulates growth hormone secretion and food intake in aged rats

Koji Toshinai, Muhtashan S. Mondal, Takuya Shimbara, Hideki Yamaguchi, Yukari Date, Kenji Kangawa, Masamitsu Nakazato *

Department of Internal Medicine, Miyazaki Medical College, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, 889-1692, Japan

Received 3 July 2006; received in revised form 21 September 2006; accepted 3 October 2006
Available online 15 November 2006

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.

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

* Corresponding author. Tel.: +81 985 85 2965; fax: +81 985 85 1869.
E-mail address: nakazato@med.miyazaki-u.ac.jp (M. Nakazato).

0047-6374/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mad.2006.10.001

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

2. Materials and methods

2.1. Animals

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

2.2. Food intake

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

2.3. GH response

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

2.4. Statistic analysis

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

3. Results

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

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

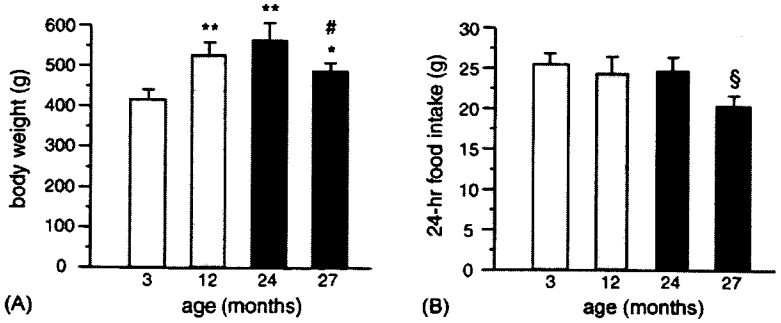

Fig. 1. Changes of body weight (A) and 24 h food intake (B) with ageing. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. 3-month-old rats, $^{\#}P < 0.05$ vs. 24-month-old, $^{\S}P < 0.01$ vs. 3-, 12-, or 24-month-old rats.

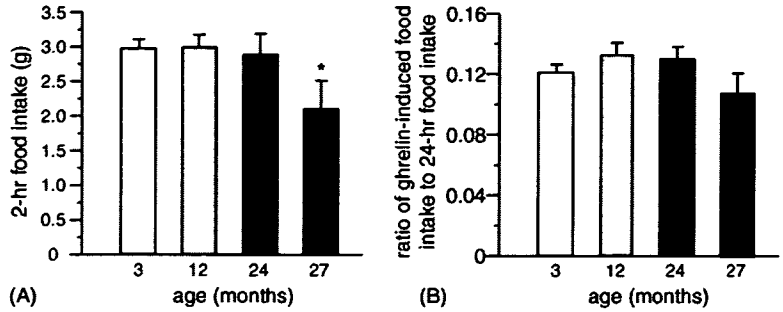

Fig. 2. (A) Effect of iv administration of ghrelin (10 nmol/kg body weight) on 2 h food intake in 3-, 12-, 24- and 27-month-old rats. $^{*}P < 0.01$ vs. 3-, 12-, or 24-month-old rats. (B) No effect of ageing on the ration of ghrelin-induced 2 h food intake to 24 h food intake.

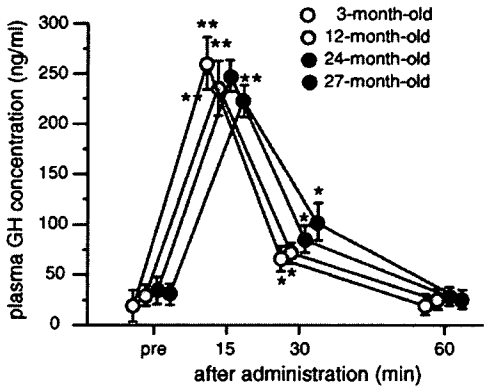

Fig. 3. Effect of iv administration of ghrelin (10 nmol/kg body weight) on the plasma GH concentration in 3-, 12-, 24- and 27-month-old rats. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. pre-administration.

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

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

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

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

4. Discussion

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

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

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

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

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

Acknowledgements

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

References

Abribat, T., Deslauriers, N., Brazeau, P., Gaudreau, P., 1991. Alterations of pituitary growth hormone-releasing factor binding sites in aging rats. Endocrinology 128, 633–635.

Aloi, J.A., Gertz, B.J., Hartman, M.L., Huhn, W.C., Pezzoli, S.S., Wittreich, J.M., Krupa, D.A., Thorner, M.O., 1994. Neuroendocrine responses to a novel growth hormone secretagogue, L-692, 429, in healthy older subjects. J. Clin. Endocrinol. Metab. 79, 943–949.

Anawalt, B.D., Merriam, G.R., 2001. Neuroendocrine aging in men. Andropause and somatopause. Endocrinol. Metab. Clin. North Am. 30, 647–649.

Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Ueno, N., Makino, S., Fujimiya, M., Niijima, A., Fujino, M.A., Kasuga, M., 2001. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120, 337–345.

Baixeras, E., Jeay, S., Kelly, P.A., Postel-Vinay, M.C., 2001. The proliferative and antiapoptotic actions of growth hormone and insulin-like growth factor-1 are mediated through distinct signaling pathways in the Pro-B Ba/F3 cell line. Endocrinology 142, 2968–2977.

Baldanzi, G., Filigheddu, N., Cutrupi, S., Catapano, F., Bonissoni, S., Fubini, A., Malan, D., Baj, G., Granata, R., Broglio, F., Papotti, M., Surico, N., Bussolino, F., Isgaard, J., Deghenghi, R., Sinigaglia, F., Prat, M., Muccioli, G., Ghigo, E., Graziani, A., 2004. Ghrelin and de-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J. Cell Biol. 159, 1029–1037.

Baumgartner, R.N., Koehler, K.M., Gallagher, D., Romero, L., Heymsfield, S.B., Ross, R.R., Garry, P.J., Lindeman, R.D., 1998. Epidemiology of sarcopenia among the elderly in New Mexico. Am. J. Epidemiol. 147, 755–763.

Bedendi, I., Allatti, G., Marcantoni, A., Malan, D., Catapano, F., Ghe, C., Deghenghi, R., Ghigo, E., Muccioli, G., 2003. Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. Eur. J. Pharmacol. 476, 87–95.

Broglio, F., Benso, A., Castiglioni, C., Gottero, C., Prodam, F., Destefanis, S., Gauna, C., van der Lely, A.J., Deghenghi, R., Bo, M., Arvat, E., Ghigo, E., 2003. The endocrine response to ghrelin as a function of gender in humans in young and elderly subjects. J. Clin. Endocrinol. Metab. 88, 1537–1542.

Ceda, G.P., Valenti, G., Butturini, U., Hoffman, A.R., 1986. Diminished pituitary responsiveness to growth hormone-releasing factor in aging male rats. Endocrinology 118, 2109–2114.

Chapman, I.M., Bach, M.A., Van Cauter, E., Farmer, M., Krupa, D., Taylor, A.M., Schilling, L.M., Cole, K.Y., Skiles, E.H., Pezzoli, S.S., Hartman, M.L., Veldhuis, J.D., Gormley, G.J., Thorner, M.O., 1996. Stimulation of the growth hormone (GH)-insulin-like growth factor-I axis by daily oral administration of a GH secretagogue (MK-0677) in healthy elderly subjects. J. Clin. Endocrinol. Metab. 81, 4249–4257.

Date, Y., Murakami, N., Kojima, M., Kuroiwa, T., Matsukura, S., Kangawa, K., Nakazato, M., 2000. Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. Biochem. Biophys. Res. Commun. 275, 477–480.

Date, Y., Toshinai, K., Koda, S., Miyazato, M., Shimbara, T., Tsuruta, T., Niijima, A., Kangawa, K., Nakazato, M., 2005. Peripheral interaction of ghrelin with cholecystokinin on feeding regulation. Endocrinology 146, 3518–3525.

Delhanty, P.J., van der Eerden, B.C., van der Velde, M., Gauna, C., Pols, H.A., Jahr, H., Chiba, H., van der Lely, A.J., van Leeuwen, J.P., 2006. Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. J. Endocrinol. 188, 37–47.

Englander, E.W., Gomez, G.A., Greeley Jr., G.H., 2004. Alterations in stomach ghrelin production and in ghrelin-induced growth hormone secretion in the age rat. Mech. Ageing Dev. 125, 871–875.

Hallfrisch, J., Muller, D., Drinkwater, D., Tobin, J., Andres, R., 1990. Continuing diet trends in men: the Baltimore Longitudinal Study of Aging (1961–1987). J. Gerontol. 45, M186–M191.

Ho, K.Y., Evans, W.S., Blizzard, R.M., Veldhuis, J.D., Merriam, G.R., Samojlik, E., Furlanetto, R., Rogol, A.D., Kaiser, D.L., Thorner, M.O., 1987. Effects of sex and age on the 24 h profile of growth hormone secretion in man: importance of endogenous estradiol concentrations. J. Clin. Endocrinol. Metab. 64, 51–58.

Koehler, K.M., 1994. The New Mexico Aging Process Study. Nutr. Rev. 8, S34.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a novel growth hormone releasing acylated peptide from stomach. Nature 402, 656–660.

Lamberts, S.W.J., van den Beld, A.W., van den Lely, A.J., 1997. The endocrinology of aging. Science 278, 419–424.

Lawrence, C.B., Snape, A.C., Baudoin, F.M., Luckman, S.M., 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. Endocrinology 143, 155–162.

MacIntosh, C.G., Andrews, J.M., Jones, K.L., Wishart, J.M., Morris, H.A., Jansen, J.B., Morley, J.E., Horowitz, M., Chapman, I.M., 1999. Effects of age on concentrations of plasma cholecystokinin, glucagon-like peptide 1, and peptide YY and their relation to appetite and pyloric motility. Am. J. Clin. Nutr. 69, 999–1006.

MacIntosh, C., Moorley, J.E., Chapman, I.M., 2000. The anorexia of aging. Nutrition 16, 983–995.

MacIntosh, C.G., Morley, J.E., Wishart, J., Morris, H., Jansen, J.B., Horowitz, M., Chapman, I.M., 2001. Effect of exogenous cholecystokinin (CCK)-8 on food intake and plasma CCK, leptin, and insulin concentrations in older and young adults: evidence for increased CCK activity as a cause of the anorexia of aging. J. Clin. Endocrinol. Metab. 86, 5830–5837.

Minisola, S., Pacitti, M.T., Scarda, A., Rosso, R., Romagnoli, E., Carnevale, V., Scarnecchia, L., Mazzuoli, G.F., 1993. Serum ionized calcium, parathyroid hormone and related variables: effect of age and sex. Bone Miner. 23, 183–193.

Morley, J.E., 2001a. Decreased food intake with aging. J. Gerontol. A Biol. Sci. Med. Sci. 56, 81–88.

Morley, J.E., 2001b. Anorexia, sarcopenia, and aging. Nutrition 17, 660–663.

Morley, J.E., Thomas, D.R., 1999. Anorexia and aging: pathophysiology. Nutrition 15, 499–503.

Muccioli, G., Tschöp, M., Papotti, M., Papotti, M., Deghenghi, R., Heiman, M., Ghigo, E., 2002. Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. Eur. J. Pharmacol. 440, 235–254.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., Matsukura, S., 2001. A role for ghrelin in the central regulation of feeding. Nature 409, 194–198.

Rigamonti, A.E., Pincelli, A.I., Corra, B., Viarengo, R., Bonomo, S.M., Galimberti, D., Scacchi, M., Scarpini, E., Cavagnini, F., Muller, E.E., 2002. Plasma ghrelin concentrations in elderly subjects: comparison with anorexic and obese patients. J. Endocrinol. 175, R1–R5.

Sandstrom, O., Mahdavi, J., el-Salhy, M., 1999. Age-related changes in antral endocrine cells in mice. Histol. Histopathol. 14, 31–36.

Shintani, M., Ogawa, Y., Ebihara, K., Aizawa-Abe, M., Miyanaga, F., Takaya, K., Hayashi, T., Inoue, G., Hosoda, K., Kojima, M., Kangawa, K., Nakao, K., 2001. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50, 227–232.

Sonntag, W.E., Hylka, V.W., Meites, J., 1983. Impaired ability of old male rats to secrete growth hormone in vivo but not in vitro in response to hpGRF(1–44). Endocrinology 113, 2305–2307.

Spik, K., Sonntag, W.E., 1989. Increased pituitary response to somatostatin in aging male rats: relationship to somatostatin receptor number and affinity. Neuroendocrinology 50, 489–494.

Sullivan, D.H., 1998. The role of nutrition in increased morbidity and mortality. Clin. Geriatr. Med. 11, 661–674.

Toshinai, K., Yamaguchi, H., Sun, Y., Smith, R.G., Yamanaka, A., Sakurai, T., Date, Y., Mondal, M.S., Shimbara, T., Kawagoe, T., Murakami, N., Miyazato, M., Kangawa, K., Nakazato, M., 2006. Des-acyl Ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. Endocrinology 147, 2306–2314.

Tschöp, M., Smiley, D.L., Heiman, M., 2000. Ghrelin induces adiposity in rodents. Nature 407, 908–913.

Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.G., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G., Ghatei, M.A., Bloom, S.R., 2000. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141, 4325–4328.

Wren, A.M., Small, C.J., Abbott, C.R., Dhillo, W.S., Seal, L.J., Cohen, M.A., Batterham, R.L., Taheri, S., Stanley, S.A., Ghatei, M.A., Bloom, S.R., 2001. Ghrelin causes hyperphagia and obesity in rats. Diabetes 50, 2540–2547.

ZOOLOGICAL SCIENCE 24: 577–587 (2007)

Natriuretic Peptides in Cetaceans: Identification, Molecular Characterization and Changes in Plasma Concentration After Landing

Tadaomi Naka[1]*†, Etsuko Katsumata[2], Kazuki Sasaki[3], Naoto Minamino[3], Motoi Yoshioka[4] and Yoshio Takei[1]

[1]*Laboratory of Physiology, Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan*
[2]*International Marine Biological Research Institute, Kamogawa Sea World, Kamogawa, Chiba 296-0041, Japan*
[3]*Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan*
[4]*Laboratory of Fish Culture, Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan*

Dolphins are aquatic animals free from gravity, and this may have imposed significant changes in their cardiovascular status and its hormonal regulation compared with terrestrial animals. This study molecularly characterized two major cardiovascular hormones, atrial and B-type natriuretic peptides (ANP and BNP) and measured their changes in dolphin plasma concentrations in relation to the cardiovascular status of the animal. We initially identified ANP and BNP in three species of dolphins (*Lagenorhynchus obliquidens*, *Phocoenoides dalli* and *Tursiops truncatus*). ANP precursors are highly conserved in most mammals, but dolphin BNP precursors were more variable. In molecular phylogenetic analyses, dolphin ANP and BNP precursors grouped with those of artiodactyls, particularly to the camel peptides. The chromatographic characterization of tissue and plasma molecular forms using specific radioimmunoassays showed that the predominant ANP and BNP in the atrium are prohormone and mature peptide, respectively, whereas mature ANP and BNP are circulating in the dolphin blood. A mass spectrometric analysis showed that atrial BNP consists of 26 amino acids, rather than the 32-amino-acid form detected in other mammals. Finally, changes in plasma ANP and BNP concentrations were examined in captive bottlenose dolphins (*Tursiops truncatus*) after their pool was drained. Plasma ANP and BNP concentrations did not change after landing, unlike terrestrial mammals. Plasma angiotensin II and cortisol concentrations did not change either, showing minor stress after landing. Since landed dolphins show a different cardiovascular status on land than terrestrial mammals, plasma ANP and BNP concentrations seem to reflect the cardiovascular status characteristic of dolphins.

Key words: atrial natriuretic peptide, B-type natriuretic peptide, molecular evolution, cardiovascular regulation, marine mammals

INTRODUCTION

Atrial and B-type natriuretic peptides (ANP and BNP) are cardioprotective hormones secreted from the heart in response to cardiac distension induced by increased venous return (preload) and/or peripheral resistance (afterload) (Lang *et al.*, 1985; Mantymaa *et al.*, 1992). The secreted hormones then act on the cardiovascular system and the kidney to reduce these loads to the heart (Clerico *et al.*, 2006). The afterload is especially severe in terrestrial animals, as they have to circulate blood against gravity. In water, however, gravity is nullified by buoyancy. Thus, blood tends to be redistributed upward to the central part of the body, and there is increased venous return. The increased venous return then dilates the atrium, resulting in ANP secretion, in the same manner that head-out water immersion increases venous return to the heart and stimulates ANP secretion in humans (Shiraishi *et al.*, 2002) and dogs (Sondeen *et al.*, 1990). Similarly, fetuses floating in amniotic fluid are also free from gravity, and it has been shown that large amounts of ANP are synthesized and secreted from both the atrium and ventricle (Bloch *et al.*, 1986; Kikuchi *et al.*, 1987; Ito *et al.*, 1990). Therefore, immersion of terrestrial animals in water seems to profoundly increase the secretion of ANP through changes in the cardiovascular balance.

* Corresponding author. Phone: +81-972-33-1405;
Fax : +81-972-33-1404;
E-mail: cetartiodactyla@gmail.com
† Present address: Nippon Suisan Kaisha, Ltd. Marine Biological Technology Center, Saiki, Oita 876-1204, Japan
doi:10.2108/zsj.24.577

In this context, it is intriguing to examine how the structure and function of the natriuretic peptide system has undergone adaptive evolution in aquatic mammals such as cetaceans (whales, dolphins and porpoises), which returned to an aquatic existence more than 50 million years ago. The prominent feature of the cetaceans in terms of cardiovascular regulation is the repetitive diving to significant depths during migration or foraging, which decreases the heart rate to a few beats/min, a phenomenon known as diving bradycardia. However, the heart rate quickly returns to normal during surfacing (Elsner *et al.*, 1966). Thus, because of a gravity-free existence in water and frequent diving behavior, cetaceans may have developed unique mechanisms for cardiovascular regulation during the transition to aquatic life. Concomitantly, major cardiovascular hormones, ANP and BNP, may have evolved as part of this cardiovascular adjustment. However, information about natriuretic peptides in marine mammals is strictly limited at both the structural and functional levels. The only studies thus far reported were by Zenteno-Savin and Castellini (1998a, b), who found increased plasma ANP and decreased angiotensin II (ANG II) and vasopressin concentrations during sleeping apnea in northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups.

In this study, we initially isolated cDNAs encoding ANP and BNP from the heart of three cetacean species, the first time this has been done in aquatic mammals. We then established a specific radioimmunoassays (RIAs) for dolphin ANP and BNP and determined their tissue and plasma concentrations for comparison with terrestrial mammals. In addition, we determined the molecular forms of ANP and BNP in the heart and plasma by different types of high performance liquid chromatography (HPLC) combined with RIA, and by mass spectrometry using semi-purified peptides. Finally, we examined the changes in plasma ANP and BNP concentrations after landing in trained bottlenose dolphins, along with another major cardiovascular hormone, ANG II, and a stress-related hormone, cortisol.

MATERIALS AND METHODS

Samples

All experiments reported here were performed in accordance with the Manual for Animal Experiments of the University of Tokyo and approved by the Committee for Animal Experiments of the University of Tokyo. Heart tissue of the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*), Dall's porpoise (*Phocoenoides dalli*), and the bottlenose dolphin (*Tursiops truncatus*) was excised from the bodies of stranded animals or those in aquaria after natural death, or obtained from fisherman within 48 h after capture. Tissues were immediately frozen and stored at –80°C until use.

Blood samples were collected from the bottlenose dolphins kept at Kamogawa Sea World, Chiba, Japan. They were trained to raise their tail flukes for blood collection from the superficial fluke veins (a husbandry blood-sampling method). Blood was withdrawn into a syringe containing 1 mg/ml 2K-EDTA and immediately centrifuged at 700×g for 10 min at 4°C, and plasma was stored at –20°C until use.

cDNA cloning

Total RNA was extracted from the heart using ISOGEN (Nippon Gene, Toyama). Poly $(A)^+$ RNA was separated from total RNA using Oligotex-dT30 (Japan Synthetic Rubber, Tokyo). The cDNA pools were prepared from 0.5 μg of Poly $(A)^+$ RNA using a SMART cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, USA). Partial cDNAs of ANP and BNP were amplified by 3' rapid amplification of cDNA ends (RACE) using primers CDS III/3' and AB-S2 (Table 1). Amplification by PCR was performed using Ex Taq DNA polymerase (TaKaRa, Tokyo) under the following conditions: 94°C for 30 s; 40 cycles of 94°C for 60 s, 55°C for 30 s, and 72°C for 90 s; and 72°C for 7 min. After 3' RACE, 5' RACE was performed using a 5' PCR primer and gene-specific primers (ANP, LoCdANP-A1 and -A2; BNP, PdBNP-A1 and -A2) (Table 1). Finally, the full coding region was amplified using 3' primer CDS III and specific primers designed in the 5' untranslated region (ANP, LoCdANP-S1 and -S2; BNP, PdBNP-S1 and -S2) (Table 1). To increase fidelity and reliability, the final amplification was performed with Advantage 2 DNA Polymerase Mix (Clontech Laboratories) from non-amplified first-strand cDNAs. All amplified products were subcloned into pT7blue vector (Novagen, Madison, USA) and sequenced with a 3130 DNA sequencer (Applied Biosystems, Foster City, USA).

Table 1. Primer sequences used for cDNA cloning and tissue expression

Name	Sequence
AB-S2	GGCTGCTTTGGGSGNMGRATNGAYCGNAT
LoCdANP-S1	AAASCAGAGRGGAGMRAGAAG
LoCdANP-S2	CCKYYCYTTGACCRACGCCAG
LoCdANP-S3	ACCTGATGGATTTCAAGAACTTGCTGG
LoCdANP-A1	TGAGGGTCTCTTGAAGTCTG
LoCdANP-A2	ATGTGAGAAATGTTGACAGGAGGCTG
LoCdANP-A4	ATGGACAGGATTGGAGCCCAGAG
PdBNP-S1	TGAGACGTGAACCGGGACCCAGCG
PdBNP-S2	AGCGGCAGCGGCGGCAACCTCTCT
PdBNP-S3	GTCTACGAGGCAGGGTCTTGGAG
PdBNP-A1	GTCCTGGCTGCATCTGGTTCT
PdBNP-A2	TCCCCTTTGAAGCAACTCCTAT
PdBNP-A3	ATCCGGTCCAGCCTCCGCCCAAAGCAG
GAPDH-S3	TACATGGTCTACATGTTCCAGTATGA
GAPDH-A4	ACTCACTCTTCTACCTTTGATGCTG

Molecular phylogenetic analysis

Deduced precursor sequences of dolphin ANP and BNP were aligned with those of other mammalian species using ClustalX version 1.83 followed by manual adjustments. Molecular phylogenetic trees were constructed by the Bayesian method with the JTT + Γ substitution model, using MrBayes version 3.1.2 software (Jones *et al.*, 1992; Ronquist and Huelsenbeck, 2003). We ran four separate Markov chains for 400,000 generations and sampled them every 100 generations to create a posterior probability distribution of 4,000 trees. The first 1,000 trees were discarded as burn-in before stabilization, and a 50% majority-rule consensus tree was constructed from the subsequent trees.

Tissue distribution of ANP and BNP

To examine the tissue expression of ANP and BNP mRNAs, total RNA was extracted from the atrium, ventricle, and kidney of three Dall's porpoises. One microgram of total RNA was reverse-transcribed using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, USA) as described previously (Takei *et al.*, 2001). For PCR, 25 μl of ExTaq buffer containing 0.5 μl cDNA, 0.2 mM dNTPs, and 0.5 μM each primer (LoCdANP-S3 and A4 for ANP; PdBNP-S3 and -A3 for BNP) (Table 1) were run for 35 cycles of 94°C for 60 s, 65°C for 30 s, and 72°C for 90 s. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

transcripts were amplified with 30 cycles of the same conditions but at an annealing temperature of 55°C using the GAPDH-S3 and -A4 primer set (Table 1). Amplified products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

Extraction of immunoreactive ANP and BNP from tissues was carried out according to our previous method (Takei *et al.*, 1989). In brief, tissue was pulverized, boiled in distilled water, acidified with acetic acid, homogenized, and centrifuged at 19,800×g for 30 min at 4°C. The supernatant was subjected to acetone precipitation (66.6%), centrifuged as above, and lyophilized. Samples were reconstituted with RIA buffer (10 mM phosphate buffer, 140 mM NaCl, 40 mM 2K-EDTA, 10 mM 6-aminohexanoic acid, 0.05% (w/v) TritonX-100 and 0.1% (w/v) NaN_3, pH 7.4) containing 0.2% of BSA (RIA grade), and immunoreactive ANP and BNP concentrations were determined by the RIA for each peptide.

Molecular characterization

The molecular forms of ANP and BNP in plasma and tissues were determined by the elution position of immunoreactive ANP and BNP in reverse-phase or gel-permeation HPLC, or by mass spectrometry of semi-purified peptides. Tissue extraction was carried out as above. Plasma was acidified with acetic acid, treated with two volumes of cold acetone, and centrifuged, and the supernatant was lyophilized for use in further analyses.

Establishment of RIAs

The specific RIAs for dolphin ANP and BNP were established using antisera raised against human ANP-28 and porcine BNP-26, respectively. The amino acid sequences of human ANP-28 and porcine BNP-26 are identical to the dolphin counterparts. The RIA protocol was as described by Kaiya and Takei (1996). Serial dilution curves for dolphin tissue and plasma extracts were parallel to the standard curves of dolphin ANP and BNP (data not shown). The cross-reactivities of dolphin BNP-26 and porcine Tyr^0-CNP-22 in the RIA for ANP were 0.1 and 0.3%, respectively, and those of dolphin ANP-28 and porcine Tyr^0-CNP-22 in the RIA for BNP were 0.01 and 0.0004%, respectively. The intra- and inter-assay coefficients of variation were 9.1 and 11.7% for ANP, and 8.9 and 15.0% for BNP, respectively. The extraction efficiency was 82.7% for the ANP and 64.5% for the BNP RIA. The minimum detectable levels in RIAs were 6.1 and 8.3 fmol/ml for ANP and BNP, respectively.

HPLC analyses

Lyophilized materials were dissolved in 10% acetonitrile in 0.1% trifluoroacetic acid (TFA) and subjected to reverse-phase HPLC on an ODS-120T column (4.6×250 mm; Tosoh, Tokyo) with a linear gradient elution of 10 to 60% acetonitrile in 0.1% TFA for 40 min at a flow rate of 1.0 ml/min. Fractions collected every minute were lyophilized for RIA. Fractions that exhibited immunoreactivity were further fractionated by gel-permeation HPLC on a Superdex Peptide HR 10/30 column (GE Health-Care Bio-Sciences Corp., Piscataway, USA) with 30% acetonitrile in 0.1% TFA at a flow rate of 0.25 ml/min. Fractions collected were lyophilized and reconstituted for RIA.

Mass spectrometry

For determination of the molecular form of BNP in the atrium, the lyophilized tissue extract was condensed and desalted with Sep-Pak C18 cartridge, and further purified by ion-exchange chromatography using SP-Sephadex C-25 gel (bed volume=200 µl) equilibrated with 10 mM ammonium acetate. Step-wise elution was carried out with 800 µl each of 25, 50, 100, 150, 200, 250, 500, and 1,000 mM ammonium acetate in 10% acetonitrile (pH 6.8). The fraction containing immunoreactive BNP was subjected to reverse-phase HPLC on an ODS-100S column (4.6×250 mm; Tosoh) with a linear gradient of 20 to 50% acetonitrile in 0.1% TFA for 40 min at a flow rate of 1.0 ml/min. Finally, gel-permeation HPLC was carried out using a Superdex Peptide column with 30% acetonitrile in 0.1% TFA at a flow rate of 0.25 ml/min.

Before mass spectrometric analysis, the samples were further purified by immunoprecipitation according to the method of Sasaki *et al.* (2002), with slight modifications. The immunoreactive fractions from gel-permeation HPLC were reconstituted with 10 mM phosphate-buffered saline (PBS) containing 0.1% TritonX-100 and incubated with BNP antisera at 4°C for 4 h. The incubate was mixed with Protein A agarose beads (Exalpha Biologicals, Inc., Maynard, USA) and incubated further for 2 h at 4°C with continuous mixing. The agarose beads were washed with PBS, and immunoreactive BNP was recovered from them with 0.2% TFA. Lyophilized material was reconstituted in 4 µl of 0.1% TFA/2% acetonitrile and added onto an aliphatic reverse-phase H4 array (Ciphergen Biosystems, Fremont, USA). After drying in air, the adsorbed materials were washed twice and mixed with alpha-cyano-4-hydroxy cinnamic acid dissolved in 0.5% TFA/50% acetonitrile. Mass spectra were acquired on a PBS II surface-enhanced laser desorption ionization (SELDI) mass spectrometer (Ciphergen Biosystems). Angiotensin I (1296.5 Da) and adrenocorticotropic hormone-related peptides (2093.5 Da, 2465.7 Da, and 3659.2 Da) were used as external calibrants, with the mass accuracy of the spectrometer better than 0.05%.

Gravitational experiment

Blood samples were obtained from bottlenose dolphins raised at Kamogawa Sea World (n=5, age=4–22 years, body length=250–314 cm, body weight=190–321 kg). The animals were free of disease and taking no medication at the time of sampling. Blood was first collected into a syringe containing 1 mg/ml of 2K-EDTA from the fluke vein, in accordance with a husbandry blood-sampling method (designated as a 'floating animal'). After sampling from floating animals, all water was gradually drained from the pool, and blood was taken again from the fluke vein in 10 min (designated as a 'landed animal'). During each blood sampling, heart rate was measured manually. Plasma was separated immediately after blood sampling.

Plasma was acidified with acetic acid and centrifuged at 10,000×g for 10 min at 4°C, and the supernatant was semi-purified using a Sep-Pak cartridge. The eluate was lyophilized and reconstituted with RIA buffer containing 0.2% BSA, and ANP, BNP, and ANG II concentrations were measured by RIA. The RIA for ANGII has been described previously (Tsuchida and Takei, 1998). Plasma cortisol concentration was measured using a Cortisol EIA Kit (Cayman Chemicals, Ann Arbor, USA) to evaluate stress during the experiments. Plasma osmolality was measured with a vapor pressure osmometer (VAPRO, Wescor, Logan, USA), Na^+ concentration with an atomic absorption spectrometer (Z5300, Hitachi, Tokyo), Cl^- concentration with a chloride meter (Buchler Instrument, USA), and glucose with a commercially available kit (Wako Pure Chemical Industries, Osaka). All assays were run in duplicate or triplicate.

Statistical analyses

Data obtained from the physiological experiments were statistically analyzed by Wilcoxon's test. Statistical significance was set at $p<0.05$. All data are expressed as means±SEM.

RESULTS

cDNA cloning

Dolphin ANP and BNP cDNAs were amplified by RACE from the heart tissue of the Pacific white-sided dolphin, Dall's porpoise, and the bottlenose dolphin, except for the BNP cDNA of the bottlenose dolphin, for which only a partial cDNA including mature sequence was obtained. Precursor sequences deduced from the cDNAs are shown in Fig. 1. Each precursor contained a mature peptide with a ring structure formed by a disulfide bond at the C terminus. The