0.064-0.907) as likely to participate in clinical research currently as respondents aged 20 to 29. There was no association between current participation and either status or previous training course in clinical research.

Discussion

In this questionnaire survey of physicians at KUH, most respondents were currently participating in clinical research and felt that clinical research was necessary. As compared to physicians participating in clinical research, smaller proportions of physicians had formal training in clinical research. The majority reported a need to acquire concepts and skills regarding clinical research, especially those related to statistics. Both previous participation in and prospective participation in clinical research were positively associated with current participation in clinical research, suggesting that physicians who were accustomed to clinical research were participating in and would participate in clinical research.

Our findings indicate that the contention that "doctors (in Japan) simply don't want to take part in clinical trials"[11] is a misunderstanding. Indeed, our results indicate that if an adequate trial infrastructure is present, Japanese physicians are eager to conduct clinical research.

KUH is an important research center in Japan, and this likely explains why the rates of participation in and acknowledgement of the importance of clinical research were high among respondents. Studies have reported a wide range in the percentage of physicians participating in clinical research, from 13% to 90% [12-14]. In a questionnaire survey at Tokushima University Hospital[8], 61% of faculty had contributed to IND application trials and 58% of those wanted to participate in IND application trials, whereas in our survey at KUH, 89% of faculty reported past participation in clinical research. The difference in participation rates could be the result of different criteria of clinical research in the questionnaire. As mentioned above, many non-notified trials are carried out at KUH and other hospitals. Perhaps the rate of participation was high because, with the exception of notified trials, physicians in Japan are able to initiate clinical research with only minimal ethical oversight.

In the present study, the difficulties that physicians faced in conducting clinical research are similar to those noted in previous studies [14-16]. Paperwork was cited as a major hurdle, even though the limited number of regulatory obstacles in Japan would be expected to lessen paperwork demands. Perhaps because physicians have a low opinion of the necessity for preparing and managing study documents, they perceive extra paperwork as onerous. Therefore, we suggest that a clinical support center should be available to provide initial advice and support regarding the production and design of documents, thereby establishing good practice. Lack of time was also reported as a major hurdle. Most physicians in university hospitals in Japan are involved in both patient care and research on molecular and cellular biology including experiments with animals. Because researchers could study molecular and cellular biology on a smaller budget than clinical research, which is the evaluation of new treatment involving human subjects, they studied it since it was introduced to Japan. As a result, there are few highly skilled clinical researchers in Japan and opportunities to learn the principles and methodology of clinical research are limited for young Japanese physicians.

Physicians who are familiar with clinical research are able to conduct clinical research more easily than those who are not, as they know the guidelines and laws necessary for conducting clinical research and can use their pre-existing network of experienced research collaborators [17]. In addition, physicians who have completed clinical trials can obtain funding more easily than those who have not; however, they gain no special treatment or financial incentives [11]. As the majority of physicians indicated that obtaining a better understanding of disease was the greatest benefit of conducting clinical research, the pleasure of discovery would appear to have more than repaid them for their efforts.

In our model with respect to current participation in clinical research, the previous training in clinical research was not found to be a significant factor. As various training providers were reported in this questionnaire, the programs and the length of these training courses should be variable. Universities or university hospitals should develop a standardized training program on clinical research that physicians can learn essential knowledge before they initiate such research.

The current study did have some limitations. The most significant of these is that the clinical research referred to in this survey comprised a variety of research types, ranging from epidemiological and observational studies to clinical trials, including IND application trials. Nevertheless, the research support section that serves the university hospital assists with a variety of clinical research designs, and a commonality of needs among physicians was demonstrated in our survey. Another limitation was that the response rate was much higher among faculty than among resident, which may influence the final logistic regression analysis. In addition, this survey took place at a single institution, so the possibility for generalization is limited. However, the difficulties indicated by respondents were quite consistent with those of prior reports. Moreover, an ongoing international collaboration project is attempting to compare the status and attitudes of physicians, and to

seek strategies to promote clinical research. The results of this study have contributed much to the refinement and modification of the questionnaire used for the international attitude study. We aim to identify unique and universal problems regarding academic clinical research, and to submit them to academic societies and governing bodies in order to improve the situation. In addition, after completion of our questionnaire survey, Ethical Guidelines for Clinical Studies were just revised and enacted in April 2009. Under the revised guidelines, investigators are now required to register their trials at a public trial registry, to obtain insurance for trial subjects, and to have adequate training in clinical research. Concern for the welfare of trial subjects may have increased, but this may create another barrier to perform clinical research by requesting more paperwork and more funding for insurance for trial subjects.

Conclusions

Physicians in university hospitals need more administrative assistance and greater knowledge of the principles and techniques of clinical research, especially the concepts of biostatistics. Our results highlight the need for training in clinical research and biostatistics and the necessity for administrative assistance in the production of study documents requested by the institutional Independent Ethics Committee.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ES conceived the study and participated in the design, management, data analysis, and preparation of the manuscript. TM participated in the study design and in the preparation of the manuscript. MY participated in the study design and participant recruitment. All authors read and approved the final manuscript.

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Ghrelin reverses experimental diabetic neuropathy in mice

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ABSTRACT

Ghrelin, an acylated peptide produced in the stomach, increases food intake and growth hormone secretion, suppresses inflammation and oxidative stress, and promotes cell survival and proliferation. We investigated the pharmacological potential of ghrelin in the treatment of polyneuropathy in uncontrolled streptozotocin (STZ)-induced diabetes in mice. Ghrelin or desacyl-ghrelin was administered daily for 4 weeks after STZ-induced diabetic polyneuropathy had developed. Ghrelin administration did not alter food intake, body weight gain, blood glucose levels, or plasma insulin levels when compared with mice given saline or desacyl-ghrelin administration. Ghrelin administration ameliorated reductions in motor and sensory nerve conduction velocities in diabetic mice and normalized their temperature sensation and plasma concentrations of 8-isoprostaglandin α, an oxidative stress marker. Desacyl-ghrelin failed to have any effect. Ghrelin administration in a mouse model of diabetes ameliorated polyneuropathy. Thus, ghrelin's effects represent a novel therapeutic paradigm for the treatment of this otherwise intractable disorder.

Introduction

Polyneuropathy is the most common complication of diabetes mellitus, occurring in more than 50% of patients who have been hyperglycemic for several years [1,2]. Diabetic neuropathy causes dysfunction of small and large nerve fibers and negatively impacts quality of life in diabetic patients. Small-fiber peripheral neuropathy is characterized by burning or lancinating pain, paresthesia, hyperesthesia, deficits in pain and temperature perception, and predisposition to foot ulceration [3]. Large-fiber dysfunctions include loss of position and vibration sensation, nerve-conduction abnormalities, and distal muscle weakness.

Two major prevailing theories relate the metabolic effects of chronic hyperglycemia and the effects of ischemia on peripheral nerves to the pathogenesis of diabetic neuropathy [4,5]. Oxidative stress is also implicated in its etiology [6]. A number of different agents from diverse chemical classes have entered clinical trials for the treatment of metabolic abnormalities in diabetic polyneuropathy, but none are yet approved for clinical use.

Ghrelin is a 28-amino-acid peptide initially isolated from human and rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) [7]. Ghrelin is predominantly produced by a distinct type of endocrine cell of the gastric oxyntic glands [8] and acts on the pituitary to stimulate GH release and on the hypothalamus to enhance food intake [7,9,10]. Ghrelin peptides exist in two major molecular forms, *n*-octanoylated ghrelin and des-*n*-octanoyl ghrelin (desacyl-ghrelin) [7]. Acylation at the third amino acid residue is necessary for the binding of ghrelin to the GHS-R; thus, the acylated form was designated as ghrelin in the original description [7]. The wide distribution of GHS-R in the nervous system, visceral organs, skin, bone, and blood vessels suggests a potentially more broad array of actions for ghrelin. In fact, ghrelin also has cardiovascular effects, increases gastric movement and gastric acid secretion, suppresses sympathetic nerve activity, and regulates glucose metabolism (reviewed in [11]).

The present study is aimed to investigate the efficacy of ghrelin on the alleviation of diabetic peripheral neuropathy induced by streptozotocin (STZ) in mice. We here administered ghrelin daily for 4 weeks after STZ-induced diabetic polyneuropathy had developed. The most useful rodent model of diabetic polyneuropathy should exhibit the key features present in human pathology [2–4], including electrophysiological measures of nerve impairment and sensory loss. Electrophysiological measures of nerve impairment are the "gold standard" for determining motor and sensory nerve function. We studied motor and sensory nerve conduction velocities (NCVs) of the sciatic nerve. We examined sensory impairment by quantitative assessment of thermal sensitivity in the hot plate test. We also

Abbreviations: 8-iso-PGF2α, 8-isoprostaglandin α; MCV, motor nerve conduction velocity; NCV, nerve conduction velocity; SCV, sensory nerve conduction velocity; STZ, streptozotocin.

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examined the effect of ghrelin administration on oxidative stress in this setting.

Materials and methods

Animals. We used 6-week-old male C57BL/6N mice weighing 15–17 g (Charles River Japan Inc., Numazu, Japan). Animals were housed individually at constant room temperature (23 ± 1 °C) in a 12-h light (08:00–20:00 h)/12-h dark cycle and were provided standard laboratory chow and water *ad libitum*. After a 24-h fast, mice were given a single intraperitoneal injection of STZ (Sigma–Aldrich, Inc., St. Louis, MO, 140 mg/kg body weight), freshly dissolved in sodium chloride at 10:00. Control mice were administered the buffer only. We measured body weights, one-day food intake, and blood glucose concentrations one day before STZ injection. Glucose levels were measured with a diagnostic kit (Ascensia Dexter-II, Bayer HealthCare AG, Leverkusen, Germany) using blood samples obtained at 21:00–22:00 from tail vein punctures in mice anesthetized by intraperitoneal injection of pentobarbital (Nembutal, 0.1 ml/mouse, Abott Co., North Chicago, IL). We also determined the motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV) of the right sciatic nerve as described below. Animals with blood glucose concentrations of greater than 17 mmol/L 3 days after STZ injection were used in this study. All diabetic animals were maintained without insulin. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Ghrelin administration. An experimental protocol of the study is outlined in Fig. 1. Four groups of 10 mice were used: the first group was administered saline (saline), the second group human ghrelin (300 nmol/kg BW/200 μL saline) (ghrelin), the third group human desacyl-ghrelin (300 nmol/kg BW/200 μL saline) (desacyl-ghrelin), and the fourth group saline without STZ treatment (controls). Eight weeks after STZ treatment or control vehicle administration, peptides or saline was administered intraperitoneally twice a day (06:00 and 18:00) for 4 weeks. We measured body weights, one-day food intake, blood glucose concentrations, and the sciatic MCV and SCV before STZ treatment and at 8, 10, and 12 weeks after STZ treatment.

Electrophysiology. Animals were anesthetized with Nembutal, and their body temperature was maintained automatically at rectal temperature of 37.5–37.9 °C by the use of a heat pad. The right sciatic nerve was stimulated (5–10 V, 0.05 ms single square-wave pulses) proximally at the level of the sciatic notch and distally at the level of the ankle with a sub-dermal needle electrode (NE-2235, NIHON KOHDEN CORP., Tokyo, Japan) [12,13]. Compound muscle action potentials were recorded from the ipsilateral foot between digits 2 and 3, amplified, stored, and displayed on a computer. SCV was determined similarly, using the same stimulating and recording electrode pairs by measuring the latency difference of the H-reflex [12]. Averaged distal and proximal motor and sensory latencies from 10 separate recordings, together with the nerve length between the two stimulation sites, were used for determination of MCV and SCV. MCV and SCV were calculated by dividing the distance between the two stimulation sites by the latency difference.

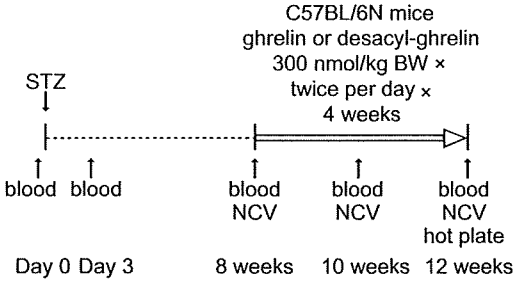

Fig. 1. Schematic representation of the experimental schedule.

Hot plate test. A hot plate test was performed after the last administration of ghrelin. Each animal was habituated to the test apparatus for 3 days before the test. The mice were placed on a hot plate maintained at 55 °C, and the latency to licking front or hind paws was monitored with a video camera and recorded on videotape [14]. Latency was analyzed by two hidden examiners.

Insulin and 8-iso-PGF2α measurements. At the end of the experiment, we deprived the mice of food for 8 h and sacrificed them under anesthesia with Nembutal at 21:00–22:00. Blood was obtained for the measurement of plasma insulin with an EIA kit (Funakoshi Chemical Co., Tokyo, Japan) and 8-isoprostaglandin F2α (8-iso-PGF2α) with an 8-isoprostan EIA kit (Funakoshi Chemical Co.).

Statistical analysis. Data are expressed as means ± SE. Differences among multiple groups were determined using a one-way or repeated-measures ANOVA with Bonferroni post hoc *t* test. When two mean values were compared, analysis was performed with unpaired *t* test. $P < 0.05$ was considered significant.

Results and discussion

Daily ghrelin administration started 8 weeks after STZ treatment when diabetes developed (Fig. 1). Food intake in the three diabetic groups at 8 weeks were significantly greater than those in controls, whereas body weight gains were suppressed in all diabetic groups (Table 1). Neither ghrelin nor desacyl-ghrelin administration to diabetic mice affected body weight gain, food intake, or blood glucose concentrations. Plasma insulin concentrations in the three diabetic groups were similar and markedly lower than that in controls (Table 2). Plasma 8-iso-PGF2α concentrations were significantly increased in the saline and desacyl-ghrelin groups, but not in the ghrelin group (Table 2).

Before commencement of ghrelin administration, 8 weeks after STZ treatment, a 27–28% decrease in sciatic MCV (Fig. 2A) was observed in the three groups of diabetic mice. Two-week ghrelin administration significantly increased MCV, but desacyl-ghrelin did not. MCV in the ghrelin group reached control levels 4 weeks after ghrelin administration. Similar to MCV, a 19–22% decrease in the sural SCV was found in the three diabetic groups (Fig. 2B). Two-week ghrelin administration also increased SCV, and at 4 weeks after the start of ghrelin administration, SCV was restored to levels comparable to that of the control group.

In the hot plate test, the licking latencies of the saline and desacyl-ghrelin groups were significantly longer than those of controls (Fig. 3). The latency was improved by ghrelin administration and became similar to the control value.

Ghrelin has received considerable attention for its diverse functions, and is an attractive, therapeutic compound for the treatment of anorexia, inflammation, and cachexia associated chronic exhausting diseases in humans [15–19]. Here, we show for the first time that ghrelin alleviated experimental diabetic sensorimotor neuropathy. Both myelinated large nerve fibers and unmyelinated small nerve fibers are affected in STZ-induced diabetic neuropathy [1]. Nerve-conduction studies are usually abnormal when large, myelinated fibers are affected. Unmyelinated, small-fiber dysfunction causes decreased sensation of pain and temperature. We evaluated the effects of ghrelin in nerve-conduction studies, hot plate test, and measurement of oxidative stress. From a therapeutic point of view, it is important to take into account that the delayed treatment with ghrelin completely restored MCV and SCV to control values in mice with established disease. Ghrelin administration restored decreased NCVs and sensory impairment in this study.

Table 1
Body weights, one-day food intake amounts, and blood glucose concentrations.

Weeks after STZ treatment		Controls	STZ treatment		
			Saline	Ghrelin	Desacyl-ghrelin
0	Body weight (g)	16.0 ± 0.8	16.3 ± 1.3	16.6 ± 2.0	16.1 ± 1.1
	One-day food intake (g)	3.0 ± 0.2	3.0 ± 0.3	3.0 ± 0.3	3.0 ± 0.3
	Blood glucose (mmol/L)	7.3 ± 0.2	7.5 ± 0.3	7.6 ± 0.3	7.3 ± 0.4
8	Body weight (g)	22.5 ± 2.1	16.8 ± 2.2*	17.2 ± 1.5*	16.3 ± 2.1*
	One-day food intake (g)	3.3 ± 0.4	7.1 ± 0.5*	6.8 ± 1.0*	6.8 ± 2.1*
	Blood glucose (mmol/L)	7.8 ± 0.6	28.1 ± 1.4*	27.3 ± 1.3*	31.7 ± 2.1*
10	Body weight (g)	24.5 ± 3.7	16.7 ± 1.3*	16.0 ± 2.1*	17.9 ± 2.4*
	One-day food intake (g)	3.4 ± 0.5	6.9 ± 0.5*	6.8 ± 0.5*	7.2 ± 1.1*
	Blood glucose (mmol/L)	7.7 ± 0.3	26.2 ± 1.0*	29.8 ± 2.0*	25.1 ± 1.1*
12	Body weight (g)	28.6 ± 3.2	18.1 ± 3.5*	18.6 ± 3.5*	17.9 ± 2.3*
	One-day food intake (g)	3.8 ± 0.5	7.0 ± 1.1*	7.0 ± 0.8*	7.1 ± 0.4*
	Blood glucose (mmol/L)	7.6 ± 0.4	26.9 ± 1.7*	28.3 ± 1.6*	29.6 ± 1.5*

Data are means ± SE. *$P < 0.01$ controls. STZ + ghrelin not significant versus STZ + saline or STZ + desacyl-ghrelin in all results.

Table 2
Plasma concentrations of insulin and 8-iso-PGF2α at the end of the experiment.

	Controls	STZ treatment		
		Saline	Ghrelin	Desacyl-ghrelin
Insulin (μU/ml)	15.93 ± 0.32	0.35 ± 0.05*	0.34 ± 0.07*	0.33 ± 0.08*,§
8-iso-PGF2α (μg/ml)	33.5 ± 8.5	52.6 ± 12.4*	31.7 ± 8.5†	48.3 ± 7.2*,§

Date are means ± SE. *$P < 0.001$ versus controls. †$P < 0.001$ versus STZ + saline and STZ + desacyl-ghrelin. §STZ + desacyl-ghrelin not significant versus STZ + saline. Comparisons were made between groups using a one-way ANOVA with Bonferroni post hoc *t* test.

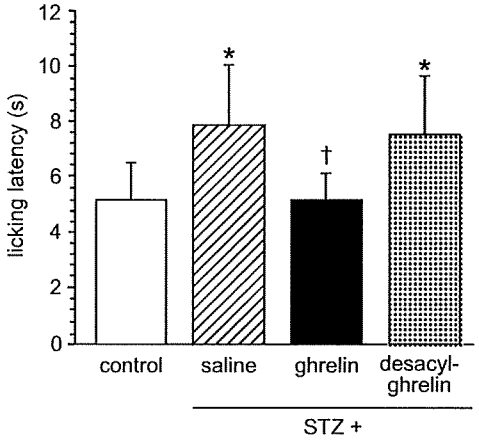

Fig. 3. Effect of ghrelin on thermal sensation. Ghrelin administration significantly improved licking latency in the hot plate test compared with saline administration. *$P < 0.01$ versus controls. †$P < 0.01$ versus STZ + saline and STZ + desacyl-ghrelin. A one-way ANOVA with Bonferroni post hoc *t* test was performed.

Multiple interacting factors including chronic hyperglycemia play a role in the pathogenesis of diabetic polyneuropathy [3–6]. There are several potential mechanisms for the effects of ghrelin on the effector phase of STZ-induced diabetic polyneuropathy. Ghrelin administration did not change body weight, food intake, blood glucose levels, or plasma insulin levels compared with the respective STZ groups without ghrelin administration, meaning that ghrelin did not improve or worsen diabetic conditions.

Diabetes compromises antioxidant defense mechanisms [20]. Ghrelin administration to human umbilical vein endothelial cells

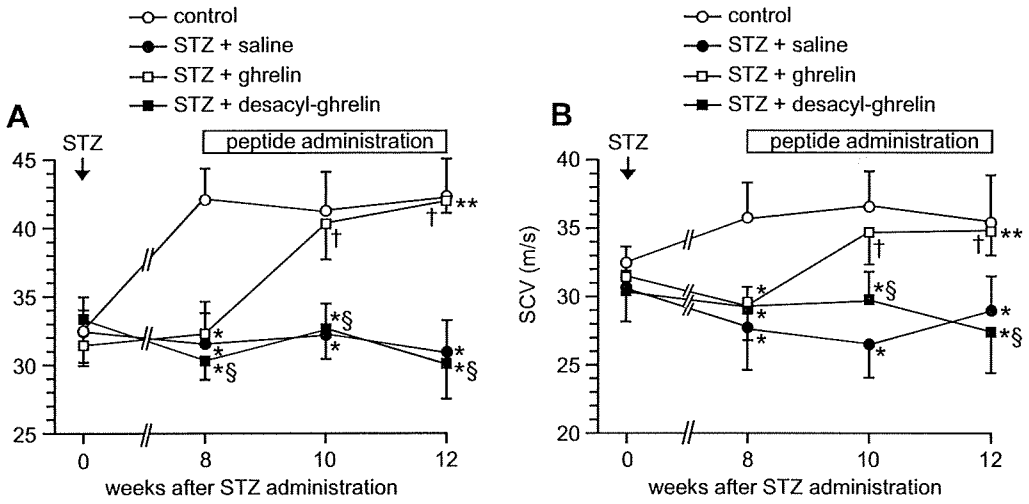

Fig. 2. Effect of ghrelin on NCV. MCV (A) and SCV (B) were reduced significantly after STZ treatment. Ghrelin administration, but not desacyl-ghrelin, ameliorated the decreases in both MCV and SCV 2 weeks after the start of administration. Data are expressed as means ± SE. *$P < 0.001$ versus controls. #$P < 0.001$ versus STZ + saline and STZ + desacyl-ghrelin. §STZ + desacyl-ghrelin not significant versus STZ + saline. Comparisons at each week were made using a one-way ANOVA with Bonferroni post hoc *t* test. Ghrelin improved the decreases in NCVs in STZ-treated diabetic mice (**$P < 0.001$ versus STZ + saline, repeated-measures ANOVA with Bonferroni post hoc *t* test for data over a period of time).

and human polymorphonuclear cells suppressed reactive oxidative species generation [21,22]. Ghrelin administration in this study ameliorated the diabetes-induced elevation of 8-iso-PGF2α levels, which may also be involved in the therapeutic effect of ghrelin.

An accumulating body of evidence has shown that desacyl-ghrelin exhibits biological activities on cardiocytes, adipocytes, myocytes, neuronal precursor cells, osteoblasts, myelocytes, and pituitary cells [23–29]. Many of these activities are associated with cell fate, such as cell survival and/or apoptosis as well as cell proliferation. Although the signaling molecules downstream of desacyl-ghrelin remain undefined, desacyl-ghrelin appears to function through an unidentified GHS-R-independent alternative pathway [23–25,28]. However, in this study, desacyl-ghrelin had no effect on reversal of diabetic neuropathy.

Our results demonstrate that ghrelin administration to a rodent model of diabetes ameliorated polyneuropathy. Ghrelin's multifaceted roles shown in this study suggest a novel therapeutic treatment for diabetic polyneuropathy. The effect of chronic administration of ghrelin to diabetic patients with polyneuropathy is under investigation in our institute.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Impaired Recovery of Blood Flow After Hind-Limb Ischemia in Mice Lacking Guanylyl Cyclase-A, a Receptor for Atrial and Brain Natriuretic Peptides

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Objective—Atrial and brain natriuretic peptides (ANP and BNP, respectively) function via guanylyl cyclase (GC)-A, resulting in diuresis, natriuresis, and blood vessel dilation. Here, we investigated the role of endogenous ANP/BNP-GC-A signaling on reparative vascular remodeling using a hind-limb ischemia model.

Methods and Results—In GC-A–deficient mice (GC-A-KO), hind-limb ischemia resulted in autoamputation or severe ulcers in 60% of mice (6/10) during the 28-day observation period. In wild-type (WT) mice, partial amputation or mild ulcers were detected in only 20% of mice (2/10). Laser Doppler perfusion imaging revealed that the recovery of blood flow in the ischemic limb was significantly inhibited in GC-A-KO mice compared with WT mice. Immunostainings with anti–PECAM-1 antibody demonstrated that, in GC-A-KO, the capillary density of the ischemic tissue was significantly diminished compared to WT. Furthermore, bone marrow transplantation showed the predominant role of GC-A on local ischemic tissue rather than on vascular progenitor cells mobilized from bone marrow during vascular remodeling. In cultured human endothelial cells, ANP treatment significantly stimulated mRNA expressions of vascular endothelial growth factor and endothelial nitric oxide synthase via Erk1/2-dependent mechanism.

Conclusion—These results suggest that endogenous ANP and BNP play important roles in reparative vascular remodeling in ischemic tissue. (***Arterioscler Thromb Vasc Biol.* 2009;29:1516-1521.**)

Key Words: atrial natriuretic peptide ■ brain natriuretic peptide ■ guanylyl cyclase-A ■ ischemia ■ mice

Vascular remodeling is critical for wound repair. In ischemic tissue, the presumed mechanism of hypoxia-induced angiogenesis involves the elevation of hypoxia-inducible factor-1α, resulting in increased expressions of growth factors, such as vascular endothelial growth factor (VEGF).[1] Angiogenic response to VEGF might involve the production of NO, as previously described in ischemic hind-limbs.[2]

The atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones that act via guanylyl cyclase (GC)-A to induce diuresis, natriuresis, and blood vessel dilation.[3] Yamahara et al found an activation of the natriuretic peptides/cyclic GMP/cyclic GMP-dependent protein kinase (PKG) pathway accelerated vascular regeneration and blood flow recovery in murine legs, for which ischemia had been induced by a femoral arterial ligation as a model for peripheral arterial diseases.[4] Recently, Park et al reported that intraperitoneal (i.p.) injection of carperitide, a recombinant human ANP, accelerated blood flow recovery with increasing capillary density in ischemic legs not only in nondiabetic mice but also in streptozotocin-induced diabetic mice, in which the blood flow recovery was significantly impaired compared with nondiabetic mice.[5] In the patients of peripheral arterial diseases, carperitide administration improved the ankle-brachial pressure index, intermittent claudication, rest pain, and ulcers.[5] These reports proved significance of the exogenous ANP and BNP in angiogenesis. However, the contributions of endogenous ANP and BNP on vascular remodeling are to be elucidated.

Here, we examined the roles of endogenous ANP and BNP in vascular remodeling using a hind-limb ischemia model.

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Methods

Animals

GC-A-knockout (GC-A-KO) mice were generated at the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center).[6] Ten-week-old male GC-A-KO mice and wild-type (WT) littermates were used in the present study.

Ligation Model

After the mice were anesthetized with pentobarbital (80 mg/kg, i.p.), hind-limb ischemia was induced as reported previously.[7] After ligating the proximal end of the left femoral artery, the distal portion of the saphenous artery was ligated, and the artery and side branches were dissected. The femoral artery and attached side branches were then excised. Sham-operated mice underwent the identical surgical procedure as described above without the actual artery ligation and excision. The mice were observed for 4 weeks. All experimental procedures conformed to the guidelines for animal experimentation of the National Cardiovascular Center and were approved by the institutional review board for animal experimentation.

Assessment of Blood Perfusion

Hind-limb blood flow was assessed with a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument) every week from the day of surgery to 4 weeks later. After blood flow was measured twice, the average flow values for the ischemic and nonischemic limbs were calculated using a computer. The LDPI index was determined as the ratio of ischemic to nonischemic hind-limb blood perfusion.

Immunohistochemistry

Ischemic hind-limb tissues 28 days after surgery were subjected to immunohistochemistry. After perfusion fixation with 4% paraformaldehyde, ischemic lower legs were embedded in OCT compound (Sakura Finetechnical) and frozen at −80°C. Cryostat sections (4 to 8 μm thick) of the tissues were stained with rat antimouse PECAM-1 antibody (Pharmingen) and alkaline phosphatase-conjugated anti–α-smooth muscle actin (α-SMA) monoclonal antibody (clone 1A4, SIGMA).

Analysis of Capillary Density

To measure capillary density, 4 random fields on 2 different sections from each mouse at ×200 magnification were photographed with a digital camera (Olympus). By computer-assisted analysis using WinRoof digital image analyzer (MITANI CORPORATION), capillary density was calculated as the mean percent positive stained area with PECAM-1 or α-SMA to the total field.

Radioimmunoassay for ANP

Radioimmunoassay (RIA) for ANP was performed as described previously.[8] Ventricle and femoral tissue were collected from mice 1 day and 7 days after operation. Tissues were diced and boiled for 10 minutes in 10 volumes of water. Glacial acetic acid was added (final concentration=1 mol/L) after cooling, and boiled tissues were homogenized. The supernatants obtained by centrifugation at 16 000g for 30 minutes were loaded onto Sep-pak C18 cartridges (Waters). After washing with 0.5 mol/L acetic acid and 0.1% trifluoroacetic acid (TFA), adsorbed materials were eluted with 60% CH_3CN containing 0.1% TFA. The eluted materials were lyophilized and submitted to RIA for ANP.

Bone Marrow Transplantation

Bone marrow transplantation (BMT) was performed as described previously.[9] Ten-week-old male WT mice and GC-A-KO mice were lethally X-irradiated with a total dose of 9 Gy (RX-650, Faxitron X-Ray Corporation). One day after, the recipient mice received unfractionated bone marrow cells (3×10^6) by cervical vein injection. Six weeks after the BMT, hind-limb ischemia was induced in the recipient mice. The reconstitution rate of the peripheral leukocytes was 80% to 85% as determined by flow cytometry.

Cell Culture, siRNA-Mediated Protein Knockdown

Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from KURABO. HUVECs were transfected 50 nmol/L siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, the cells were used for the experiments.

Real-Time PCR

Real-Time PCR was performed as described previously.[10] Total RNA was extracted from mice femoral tissue and cultured HUVECs. First-strand cDNA was synthesized using SUPERSCRIPT II Reverse Transcriptase (Invitrogen) from 2 μg of total RNA. To quantitatively examine levels of GC-A endothelial nitric oxide synthase (eNOS), VEGF, and Angiopoietin2 (Ang2) mRNAs, real-time PCR amplification using a Light Cycler system (Roche Applied Science) was performed according to the manufacturer's instructions. Known concentrations of linearized plasmids containing mice GC-A, human eNOS, human VEGF, human Ang2 cDNA were used to generate standard curves. Gene expression was normalized to GAPDH. Primers were designed on Primer Express (PE Applied Biosystems Inc) coordinates as follows:

mice GC-A forward, (GCAACCAAGAGACCACTTTTCCA); mice GC-A reverse, (CGTTTTCCGGTTCACACGTTT); mice GAPDH forward, (TGCAGTGGCAAAGTGGAGATT); mice GAPDH reverse, (TCGCTCCTGGAAGATGGTGAT); human eNOS forward, (TCGTCCCTGTGGAAAGACAAG); human eNOS reverse, (TCTCGGAGCCATACAGGATTG); human VEGF forward, (CAGCTACTGCCATCCAATCGA); human VEGF reverse, (TTTGCCCCTTTCCCTTTCC); human Ang2 forward, (GACTGCCACGGTGAATAATTCA); human Ang2 reverse, (CGTGTAGATGCCATTCGTGGT); human GAPDH forward, (TGAAGGTCGGAGTCAACGGAT); human GAPDH reverse, (ACGGTGCCATGGAATTTGC).

Western Blot Analysis

The following antibodies were purchased from Cell Signaling Technology: phospho-Erk1/2 (Thr202/204) antibody, Erk1/2 antibody, phospho-p38 MAPK (Thr180/Tyr182) antibody, phospho-SAPK/JNK (Thr183/Tyr185) antibody, phospho-Akt (Thr308) antibody, phospho-Akt (Ser473) antibody, phospho-AMPKα (Thr172) antibody, phospho-AMPKα (Ser485) antibody, phospho-eNOS (Ser1177) antibody, phospho-VASP (Ser157) antibody, phospho-VASP (Ser239) antibody, VASP antibody, GAPDH antibody. Western blot analysis was performed as described previously.[11] HUVECs and HAECs were lysed with cell lysis buffer (20 mmol/L Tris-HCl, pH7.5, 150 mmol/L NaCl, 1mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L Sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin; Cell Signaling Technology) with protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation, and total protein concentrations were determined using Bio-Rad Protein Assay. Samples were electrophoresed through a reducing SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and incubated with antibodies noted above. Protein expression was detected with a Phototope-HRP Western Blot Detection System (Cell Signaling Technology).

Results

Significance of Endogenous ANP and BNP in Postnatal Vascular Regeneration

As shown in Figure 1A, hind-limb ischemia in WT mice resulted in partial amputation of the limb and mild ulcers

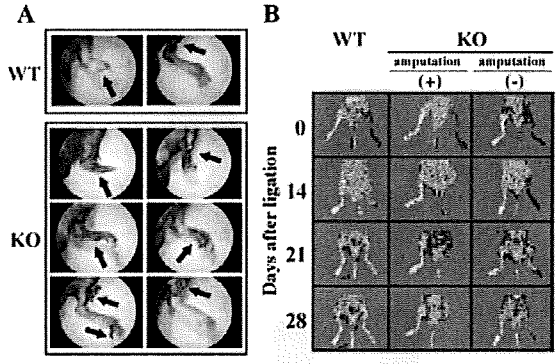

Figure 1. Contribution of GC-A–mediated signaling to vascular remodeling. A, Photographs obtained 28 days after operation in WT and GC-A-KO (KO) mice. Lesions are marked with arrows. B, Serial LDPI of hind-limb ischemia in WT and KO mice.

in 2 of 10 mice, whereas limb amputation and severe ulcers were observed in 6 of 10 GC-A-KO mice. Laser Doppler imaging revealed that, even though the rest of GC-A-KO mice did not show apparent limb lesions, blood flow recovery was significantly inhibited compared with WT mice (Figure 1B, amputation (−)). Figure 2A shows the time of course of blood flow recovery in GC-A-KO mice, which was reduced significantly compared with WT mice throughout the observation period.

Next, we evaluated capillary network formation and arteriole coverage in the ischemic tissue by immunostaining for PECAM-1 and α-SMA, respectively. As shown in Figure 2B, the PECAM-1–expressing area was significantly diminished in GC-A-KO mice compared with WT mice (3.5±0.2% versus 5.8±0.3%, $P<0.0001$). In the section, consecutive to that used in Figure 2B, the percentage of the sample area positive for α-SMA was also significantly smaller in GC-A-KO mice compared with WT mice (0.8±0.3% versus 3.9±0.7%, $P<0.01$; Figure 2C).

Importance of Local GC-A, Expressed in Ischemic Tissue in Vascular Remodeling

Recently, bone marrow (BM)-derived vascular progenitor cells (ie, EPC) have been reported to contribute to the reparative neovascularization.[12] Therefore, we next investigated the role of BM-derived cells in GC-A–mediated vascular remodeling with BMT experiments. As shown in Figure 3A, when GC-A-KO BM was transplanted to WT (BMT; KO to WT), blood flow recovery was significantly reduced compared with its control (BMT; WT to WT). Of note, inversely, when WT BM was transplanted to GC-A-KO (BMT; WT to KO), blood flow recovery was almost equal to its control (BMT; KO to KO). Next, we evaluated changes of GC-A expression level in ischemic limb tissue at day 1 and day 7 after operation using WT mice. As shown in Figure 3B, at day 1, GC-A expression level was comparable in femoral artery ligated and sham-operated group. However, at day 7, GC-A expression level was significantly increased in femoral artery ligated group (1.7-fold, $P<0.01$). These results indicate that local GC-A, expressed in ischemic limb tissue, and GC-A, expressed on BM-derived vascular progenitor cells, both play important roles in vascular remodeling.

Furthermore, we next examined whether ANP contents in the ventricle or in ischemic limb tissue were changed after ligation of femoral artery of the WT. As shown in supplemental Table I (available online at http://atvb.ahajournals.org), ANP contents in ventricle and ischemic limb tissue did not change significantly up to 7 days after ligation of the femoral artery.

ANP Stimulates eNOS and VEGF mRNA Expressions

To dissect the mechanism of ANP-mediated vascular repair promotion, we examined the changes of mRNA expression levels of eNOS, VEGF, and Ang2, all of which have been well known to contribute to angiogenesis, in cultured HUVECs after treatment of ANP. As shown in Figure 3C, ANP treatment significantly augmented eNOS (2.1-fold, $P<0.01$) and VEGF (2.0-fold, $P<0.001$) mRNA expression levels, whereas it did not change Ang2 mRNA expression level. Previous reports demonstrated that eNOS activity is regulated by phosphorylation at Ser1177.[13] Therefore, we performed Western blot analysis using a specific antibody against phospho-serine^{1177} of eNOS. As shown in Figure 3D,

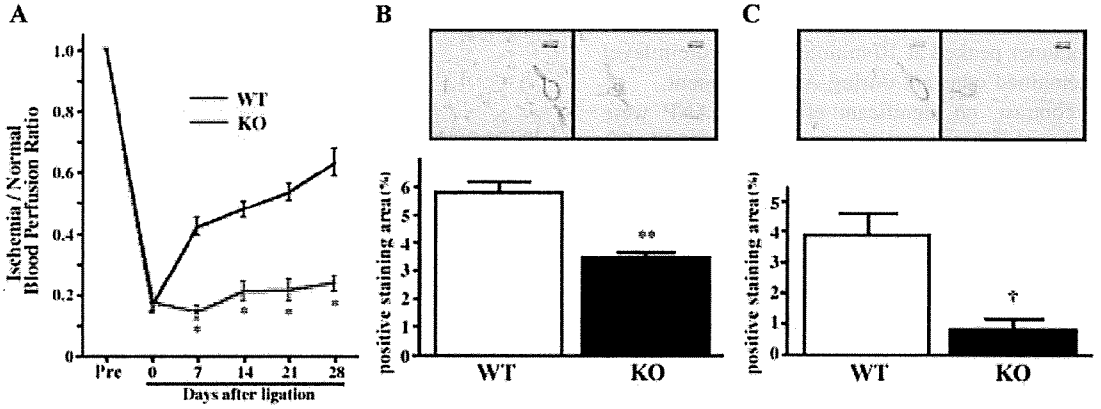

Figure 2. A, Serial quantitative analysis of the ischemic/normal hind-limb perfusion ratio in WT and GC-A-KO (KO) mice using LDPI. *$P<0.05$ vs WT. B and C, Immunostaining of ischemic hind-limbs using anti-PECAM-1 antibody (B) and anti-α-SMA antibody (C) on day 28. Values are expressed as the means±SEM. **$P<0.0001$ vs WT. †$P<0.01$ vs WT.

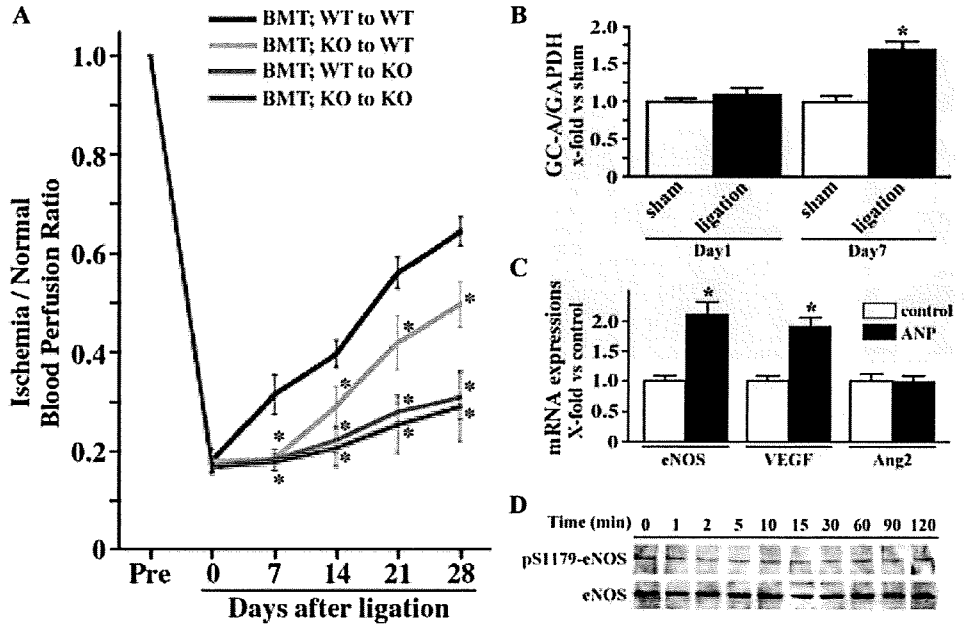

Figure 3. A, Serial quantitative analysis of ischemic/normal hind-limb perfusion ratio in WT and GC-A-KO (KO) mice using LDPI after BMT experiments. Values are expressed as the means±SEM. *$P<0.05$ vs BMT; WT to WT. B, Effect of femoral artery ligation and excision to GC-A mRNA expression at day 1 and day 7 after operation in the femoral tissue in WT mice. Values are expressed as the means±SEM. *$P<0.05$ vs sham-operation group. C, Effect of ANP (10^{-7} mol/L) treatment on eNOS, VEGF, and Angiopoietin2 (Ang2) mRNA expressions in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated ANP for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control. D, Effect of ANP (10^{-7} mol/L) treatment on eNOS phosphorylation in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated with ANP (10^{-7} mol/L) for the time indicated at the top.

ANP treatment of HUVECs did not significantly change eNOS phosphorylation status on Ser1177. Therefore, it is suggested that the effect of ANP on eNOS activation could be mediated not by protein phosphorylation but by augmentation of gene expression.

Erk Mediates ANP-Induced eNOS and VEGF mRNA Expressions

Next, we investigated downstream signals of GC-A by Western blot analysis using phospho-specific antibodies of various kinase. We stimulated cultured HUVECs with ANP and assessed phosphorylation of Erk1/2 at different time points up to 120 minutes. As shown in Figure 4A, ANP greatly enhanced Erk1/2 phosphorylation, in a time-dependent manner peaked at 10 minutes. The protein level of Erk1/2 remained constant during ANP treatment.

In clear contrast, no significant effects of ANP were demonstrated on the phosphorylation status of the p38 MAPK (Thr180/Tyr182), SAPK/JNK (Thr183/Tyr185), Akt (Thr308), Akt (Ser473), AMPKα (Thr172), AMPKα (Ser485) in the same experimental condition describe above (data not shown). We also assessed whether Erk1/2 is phosphorylated by ANP in arterial endothelial cells. As shown in Figure 4B, ANP initiated Erk1/2 phosphorylation in cultured HAECs as well.

Vasodilator-stimulated phosphoprotein (VASP) is a member of the family of actin binding regulatory proteins, which induces stress fiber formation and membrane ruffling in vascular endothelial cells.[14] As shown in Figure 4C, ANP greatly enhanced VASP phosphorylation at Ser239, which reached maximum at 5 minutes and then remained relatively constant for up to 120 minutes, whereas the VASP protein expression remained constant during treatment.

Next, we sought to determine whether the ANP-induced Erk1/2 or VASP activation contributes to the upregulation of eNOS and VEGF genes, using PD98059, an Erk1/2 inhibitor, and VASP siRNA. The protein knockdown effects of VASP siRNAs were confirmed as shown in Figure 4D. Treatment of HUVECs with PD98059 significantly reduced ANP-mediated increases of eNOS and VEGF mRNA expressions (Figure 4E and 4F). In clear contrast, VASP protein knockdown by siRNA#1 had no effect on ANP-mediated increases of the gene expressions (Figure 4G and 4H).

Discussion

In the present study, we first demonstrated that, after ligation of the femoral artery, the blood flow recovery and capillary formation were reduced in the mice deficient for GC-A, a common receptor for ANP and BNP, compared with WT mice. Second, we proved that GC-A mRNA expression was upregulated in the ischemic hind-limb tissue at day 7 after operation. Third, we demonstrated that not only local GC-A in ischemic tissue but also GC-A on BM-derived cells contributes to the vascular regeneration. We also indicated that, in cultured endothelial cells, ANP treatment increased eNOS and VEGF mRNA expressions by Erk1/2-dependent mechanism.

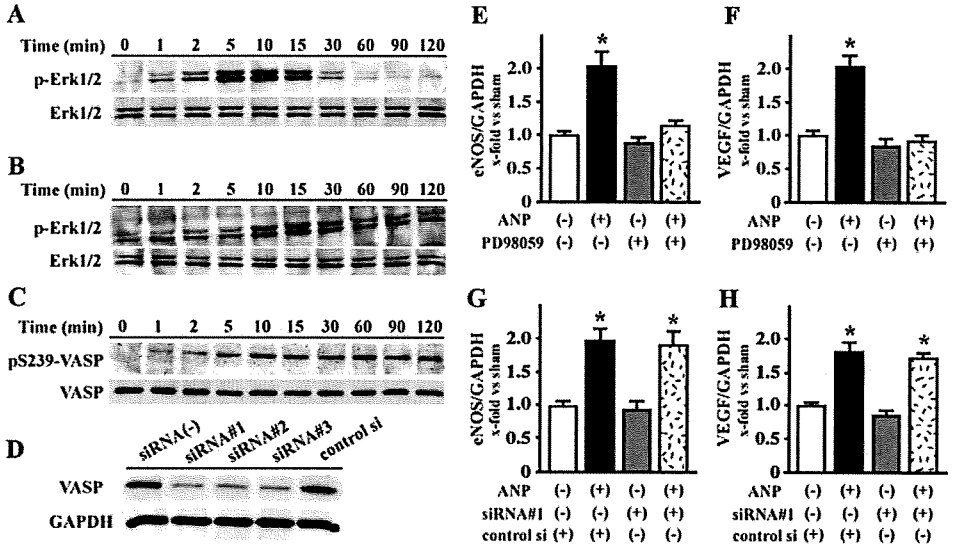

Figure 4. A–C, Effect of ANP (10^{-7} mol/L) treatment on Erk1/2 (A, B) and VASP (C) phosphorylation in HUVECs (A, C) and HAECs (B). Cells starved for 12 hours in medium containing 0.5% FCS were stimulated with ANP for the time indicated at the top. D, VASP protein knockdown effect of 3 types of siRNA in HUVECs. After siRNA transfection, cells were cultured in growth medium for 24 hours, and then starved for 12 hours in medium containing 0.5% FCS. Thereafter, cells were stimulated with ANP for 8 hours with or without siRNA. Control si means negative control siRNA. E and F, Effect of Erk1/2 inhibitor PD98059 (2×10^{-5} mol/L) on ANP (10^{-7} mol/L)-induced augmentation of eNOS (E) and VEGF (F) mRNA expressions in HUVECs. Cells starved for 12 hours in medium containing 0.5% FCS were stimulated by ANP with or without PD98059 for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control. G and H, Effect of VASP siRNA on ANP (10^{-7} mol/L)-induced augmentation of eNOS (G) and VEGF (H) mRNA expressions in HUVECs. After siRNA transfection, cells were cultured in growth medium for 24 hours and then starved for 12 hours in medium containing 0.5% FCS. Thereafter, cells were stimulated with ANP for 8 hours. Results are means±SEM of 4 independent assays. *$P<0.05$ vs control.

Because GC-A is highly expressed in vascular endothelial cells,[15] we initially hypothesized that local GC-A on the endothelial cells of ischemic tissue could play an important role in the angiogenic effect of ANP. In fact, it has been reported that ANP induces an increase in the number of cultured endothelial cells and potentiates capillary network formation in vitro.[16] However, because recent evidences have indicated that BM-derived vascular progenitor cells contribute to neovascularization of ischemic lesions,[12] and because our preliminary experiment revealed that GC-A is expressed in these cells as well, sites of angiogenic action of ANP needed to be determined. We therefore designed BMT experiments that revealed that not only local GC-A but also GC-A on the BM-derived cells contributes to the postischemic vascular regeneration. Interestingly, in the GC-A-KO background, BMT of WT to KO had no significant change in postischemic blood flow recovery compared to BMT of KO to KO, implicating that GC-A in the local ischemic tissue is necessary for the development of ANP action on BM-derived cells.

In the present study, we have examined the effect of ANP on gene expressions of eNOS and VEGF, the key roles of which in vascular regeneration were clearly demonstrated by previous excellent studies.[1,2] Importantly, incubation of cultured endothelial cells with ANP for 8 hours significantly upregulated mRNA expressions of both eNOS and VEGF and the effects of ANP were efficiently blocked by the concomitant addition of Erk1/2 inhibitor. Taken together, it is suggested that ANP induces eNOS and VEGF mRNA expressions through Erk1/2 activation, which presumably plays a significant role in the ANP-induced vascular remodeling. In the present study, we also demonstrated that ANP treatment of HUVECs strongly phosphorylates VASP, which agrees with the recent report by Chen et al.[17] Because VASP has been known to have multiple effects on the physiological processes governed by cellular actin networks, such as cell motility, migrations, angiogenesis, and vascular permeability,[18] it is suggested that ANP-mediated VASP activation could promote actin stress fiber formation and endothelial tube formation.[17] However, whether inhibition of VASP may actually affect ANP-induced vascular remodeling in vivo awaits further studies.

Because ANP and BNP both bind to and activate GC-A, it would be worth speculating which ligand is pivotal for the angiogenic property of the endogenous natriuretic peptide system. Although the binding affinity of BNP to GC-A receptor is comparable to that of ANP,[19] plasma level of ANP is approximately 20-times higher than BNP concentration in humans and animals.[20] We therefore hypothesize that, rather than BNP, ANP may play a predominate role for the vascular remodeling in vivo. However, in heart failure, plasma level of BNP becomes comparable to or even higher than that of ANP.[21] Therefore, the role of BNP on peripheral vascular repair would become more important in subjects with cardiac diseases.

In conclusion, the present study strongly suggests that endogenous ANP and BNP play important roles in reparative vascular remodeling.

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Disclosures

None.

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A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance

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[1]*Ghrelin Research Project, Translational Research Center, Kyoto University Hospital, Kyoto University Graduate School of Medicine;* [2]*Department of Medicine and Clinical Science, Endocrinology, and Metabolism, Kyoto University Graduate School of Medicine;* [3]*Clinical Research Institute for Endocrine Metabolic Diseases, National Hospital Organization, Kyoto Medical Center, Kyoto; and* [4]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*

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Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T. A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. *Am J Physiol Endocrinol Metab* 297: E802–E811, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00205.2009.—Ghrelin is a stomach-derived peptide that has growth hormone-stimulating and orexigenic activities. Although there have been several reports of ghrelinoma cases, only a few cases have elevated circulating ghrelin levels, hampering the investigation of pathophysiological features of ghrelinoma and chronic effects of ghrelin excess. Furthermore, standard transgenic technique has resulted in desacyl ghrelin production only because of the limited tissue expression of ghrelin *O*-acyltransferase, which mediates acylation of ghrelin. Accordingly, we attempted to create ghrelin promoter SV40 T-antigen transgenic (GP-Tag Tg) mice, in which ghrelin-producing cells continued to proliferate and finally developed into ghrelinoma. Adult GP-Tag Tg mice showed elevated plasma ghrelin levels with preserved physiological regulation. Adult GP-Tag Tg mice with increased plasma ghrelin levels exhibited elevated IGF-I levels despite poor nutrition. Although basal growth hormone levels were not changed, those after growth hormone-releasing hormone injection tended to be higher. These results indicate that chronic elevation of ghrelin activates GH-IGF-I axis. In addition, GP-Tag Tg mice demonstrated glucose intolerance. Insulin secretion by glucose tolerance tests was significantly attenuated in GP-Tag Tg, whereas insulin sensitivity determined by insulin tolerance tests was preserved, indicating that chronic elevation of ghrelin suppresses insulin secretion and leads to glucose intorelance. Thus, we successfully generated a Tg model of ghrelinoma, which is a good tool to investigate chronic effects of ghrelin excess. Moreover, their characteristic features could be a hint on ghrelinoma.

ghrelin; glucose metabolism

GHRELIN is a stomach-derived 28-amino acid (AA) peptide hormone with octanoyl modification of third Ser residue, which is essential for its binding to growth hormone (GH) secretagogue receptor (GHS-R) (20). There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). As far as we know, only two cases have elevated plasma ghrelin level (9, 36). However, the ghrelin-producing cells in the stomach, known as X/A-like cells, account for about 20% of the endocrine cell population in the oxyntic glands (10). It may be reasonable to estimate that far more ghrelinoma cases have been overlooked and diagnosed as nonfunctioning tumors. Hormone-producing tumors demonstrate their characteristic symptoms by chronic effects of each hormone, which may be a key symptom to making a correct diagnosis. Conversely, the characteristic symptom often tells us the chronic effects of each responsible hormone. Acute effects of ghrelin have been studied extensively by many researchers, and a wide variety of acute effects of ghrelin have been discovered, such as the regulation of growth hormone (GH) release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (23, 25, 26, 31, 33, 34). However, chronic effects of ghrelin have not been fully understood.

To understand the chronic effects of ghrelin, genetically engineered mouse models would be useful. Several groups, including ours, have developed transgenic animals in which ghrelin transgenes are driven by several different promoters (2, 4, 18, 29, 38, 41). All of these animals except for one line created by Reed et al. (29) using the neuron-specific enolase (NSE) promoter and another line recently reported by Bewick et al. (5) using the bacterial artificial chromosome produced only desacyl ghrelin rather than acylated ghrelin. Until the recent identification of ghrelin *O*-acyltransferase (GOAT), which mediates ghrelin octanoylation (40), it had been unclear how acylation of ghrelin takes place. GOAT is expressed mainly in stomach and intestine, and a small amount of GOAT is also present in pancreas (12). This limited expression area of GOAT made it impossible to create ghrelin-overproducing transgenic animals by standard procedures. When we started this study, GOAT had not yet been identified. Accordingly, we choose an approach in which an increase in the number of ghrelin-producing cells in mice would result in increased levels of circulating ghrelin. By taking this approach, we successfully obtained ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice. In these mice, ghrelin concentration elevates with age in concordance with the proliferation of ghrelin cells. The aim of this study was to elucidate the pathophysiological features of ghrelinoma and the chronic effects of ghrelin elevation.

MATERIALS AND METHODS

Animals. Two types of fusion genes comprising the 5′-flanking region of human ghrelin gene (4,085 or 1,479 bp) (19) and SV40 T-antigen were designed (Fig. 1*A*). The purified fragments (10 μg/ml) were microinjected into the pronucleus of fertilized C57/B6 mouse (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by

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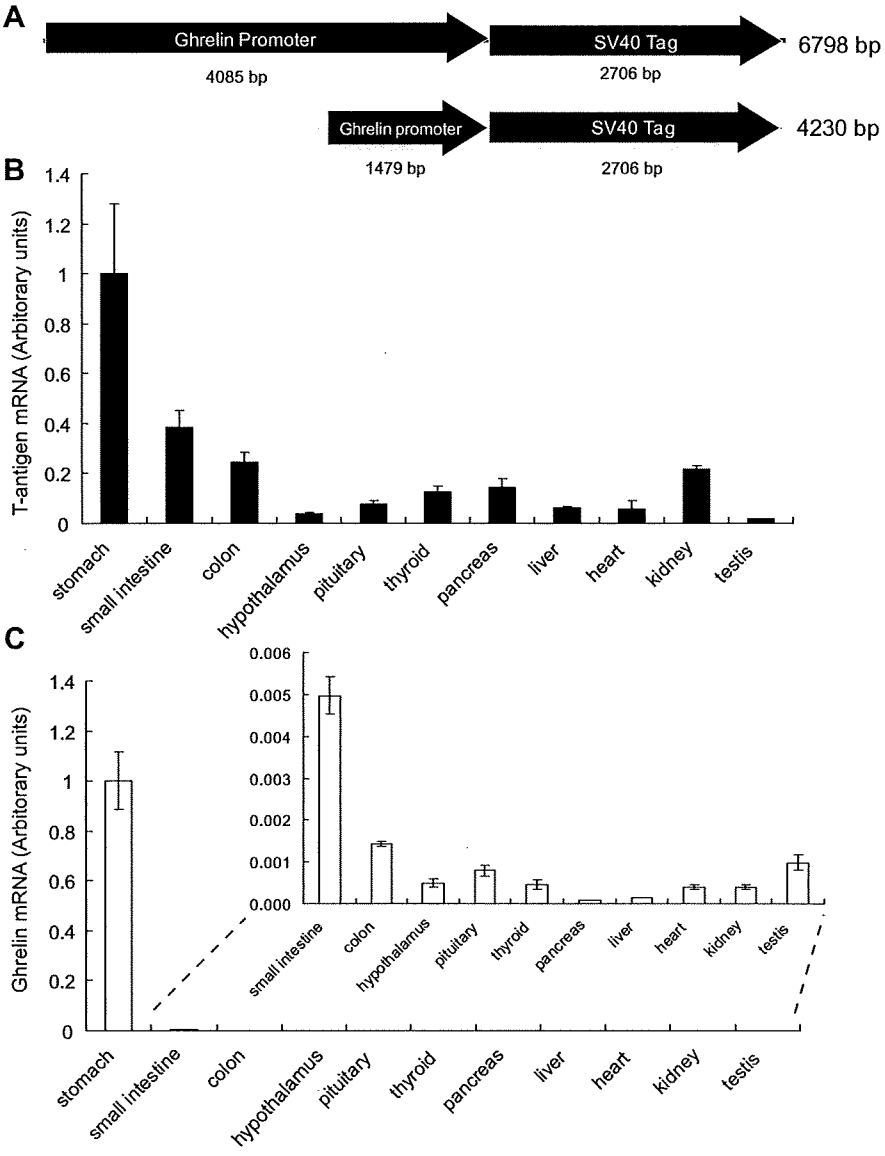


Fig. 1. Constructs of ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice and the expression levels of SV40 T-antigen mRNA in various tissues. *A*: 2 types of fusion genes comprising 5′-flanking region of human ghrelin gene (4,085 or 1,479 bp) and SV40 Tag were designed. *B*: the expression levels of SV40 T-antigen mRNA in various tissues of GP-Tag Tg mice at 6 wk of age (n = 8). SV40 T-antigen mRNA was most abundant in the stomachs of GP-Tag Tg mice. *C*: the expression levels of ghrelin mRNA in various tissues of nontransgenic littermates at 6 wk of age (n = 4).

Southern blot analysis of tail DNAs. Transgenic mice were used as heterozygotes. Animals were maintained on standard rodent food (CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) on 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.

RT-PCR and real-time quantitative RT-PCR. Total RNA was extracted using a Sepasol RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). RT-PCR was carried out with a GeneAmp 9700 using primers in Table 1 with AmpliTaq Gold PCR master mix (Applied Biosystems). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with primers and TaqMan probes or with Power SybrGreen (presented in Table 1). The mRNA expression in each gene was normalized to levels of 18S ribosomal RNA.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA), as described previously (18). Sections were incubated with anti-COOH-terminal ghrelin (AA 13-28) (1:2,000 at final dilution), anti-NH_2-terminal ghrelin (14) that recognizes the n-octanoylated portion of ghrelin (AA 1-11) (1:5,000), anti-glucagon (1:500; DAKO, Glostrup, Denmark), anti-somatostatin (1:500; DAKO), anti-gastrin (1:500; DAKO), and anti-GH (1:500; DAKO). The cell number of ghrelin-immunopositive cells was analyzed by WinRoof visual analysis software (Mitani, Fukui, Japan).

Measurements of plasma and tissue ghrelin concentrations. Collection of plasma samples was performed as reported previously (18). Plasma ghrelin and desacyl ghrelin concentrations were determined using two separate ELISA kits, an active ghrelin ELISA kit that recognizes n-octanoylated ghrelin and a desacyl ghrelin ELISA kit (both from Mitsubishi Kagaku Iatron, Tokyo, Japan) (1). Tissue ghrelin concentration was determined by radioimmunoassay (RIA) using anti-ghrelin (AA 13-28) antiserum (C-RIA) and anti-ghrelin (AA 1-11) antiserum (N-RIA), as described previously (18).

Western blot. Stomachs were boiled for 5 min in the 10-fold vol/wt of water. Acetic acid was added to each solution so that the final concentration was adjusted to 1 M, and the tissues were homogenized.

Table 1. *PCR primers and TaqMan probes*

Gene	Primer Sequence
Ghrelin	
Sense	5′-GCATGCTCTGGATGGACATG-3′
Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
TaqMan probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
NPY	
Sense	5′-TCCGCTCTGCGACACTACAT-3′
Antisense	5′-GGAAGGGTCTTCAAGCCTTGT-3′
TaqMan probe	5′-CAAGGGCTGGATCTCTTGCCATATCTCTG-3′
AgRP	
Sense	5′-GCTCCACTGAAGGGCATCA-3′
Antisense	5′-TAGCACCTCCGCCAAAGCT-3′
TaqMan probe	5′-TTCCCAGGTCTAAGTCTGAATGGCCTCA-3′
GHRH	
Sense	5′-AGGATGCAGCGACACGTAGA-3′
Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
TaqMan probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
Somatostatin	
Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
TaqMan probe	5′-CGAACCCAGCAATGGCACCCC-3′
GHS-R	
Sense	5′-CACCAACCTCTACCTATCCAGCAT-3′
Antisense	5′-CTGACAAACTGGAAGAGTTTGCA-3′
TaqMan probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′
GH	
Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
TaqMan probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH-R	
Sense	5′-GCCCTTGGAACTGTTAACCA-3′
Antisense	5′-GCAACCAGGATGGCAATAGC-3′
TaqMan probe	5′-AGCATCTCCATTGTAGCCCTCTGCGTG-3′
SV40 Tag	
Sense	5′-AAACACTGCAGGCCAGATTT-3′
Antisense with power SYBR Green	5′-AAATGAGCCTTGGGACTGTG-3′
PC1/3	
Sense	5′-AGTGGAAAAGATGGTGAATG-3′
Antisense	5′-CTCCTCATTTAGGATGTCCA-3′

NPY, neuropeptide Y; AgRP, agouti-related protein; GHRH, growth hormone (GH)-releasing hormone; GHS-R, GH secretagogue receptor; GHRH-R, GHRH receptor; PC1/3, prohormone convertase 1/3.

The supernatant was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA) preequilibrated with 0.9% NaCl after centrifugation. The cartridge was washed with 2.5 ml of 5% CH_3CN-0.1% trifluoroacetic acid and eluted with 2.5 ml 60% CH_3CN-0.1% trifluoroacetic acid. The eluate was evaporated, lyophilized, and dissolved in Novex Tricine SDS Sample Buffer (Invitrogen, Carlsbad, CA). After being heated at 85°C for 2 min, 20 mg of samples of initial weight were subjected to tricine-SDS PAGE and electroblotted to polyvinylidene fluoride membranes (Invitrogen). Transferred membranes were blocked with Immunoblock (Dainippon Seiyaku, Osaka, Japan) and then incubated with anti-COOH-terminal ghrelin antibody (1:5,000). After being washed with PBS-0.1% Tween-20, membranes were reacted with secondary antibodies and developed with ECL plus (GE Healthcare, Buckinghamshire, UK) as instructed by the manufacturer. The signal on the blot was detected with Lumino-Image Analyzer LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

Measurement of food intake. Mice were housed individually with continuous access to chow and water. Food intakes were measured by subtracting the remaining weight of the chow from that originally presented. As for measuring the food intake by ghrelin, ad libitum-fed mice were injected with ghrelin (120 or 360 μg/kg) or saline subcutaneously. Food intakes were measured for 2 h after injection.

Measurements of lean body mass, fat mass, and bone mass. Mice were anesthetized with pentobarbital sodium. Lean body mass, fat mass, and bone mass of mice were measured by an animal computed tomography system (Latheta LTC-100; Aloka, Tokyo, Japan).

Measurements of hormones and blood glucose levels. Serum GH levels were determined by a rat GH EIA kit (SPI Bio, Massy Cedex, France). Serum insulin-like growth factor I (IGF-I) levels were measured using a mouse IGF-I immunoassay kit (R & D Systems, Minneapolis, MN). Blood glucose levels were determined by glucose oxidase method using Glutest Sensor Neo (Sanwa Kagaku, Kyoto, Japan). Measurement of serum insulin concentrations was performed by ELISA using an ultrasensitive rat insulin kit (Morinaga, Yokohama, Japan).

GH-provocative test. GH-provocative test was carried out as described previously (16). Serum samples were collected at 15 min after subcutaneous injection of 180 μg/kg of GH-releasing hormone (GHRH) or 120 μg/kg of ghrelin. We choose these doses according to the results of our previous study (16).

Glucose and insulin tolerance tests. For the glucose tolerance test, after overnight fast, the mice were injected with 1.5 g/kg glucose intraperitoneally. For the insulin tolerance test, after a 4-h fast, mice were injected with 1.0 mU/g human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally. Blood was sampled from the tail vein before and 30, 60, 90, and 120 min after the injection.

Insulin release. After overnight fast, the mice were injected with 3.0 g/kg glucose intraperitoneally. Blood was sampled from the retroorbital vein at 2 and 30 min after the injection using a glass tube.

Statistical analysis. All values were expressed as means ± SE. The statistical significance of the differences in mean values was assessed by repeated-measures ANOVA or Student's *t*-test. The statistical difference in the changes of plasma ghrelin levels by feeding were assessed by paired *t*-test. Pearson's correlation coefficient analysis and simple regression were used to assess the relations between plasma ghrelin level and body weight. Difference of correlation coefficients of the regression lines obtained from GP-Tag Tg mice and nontransgenic littermates was determined by testing the *t* value.

RESULTS

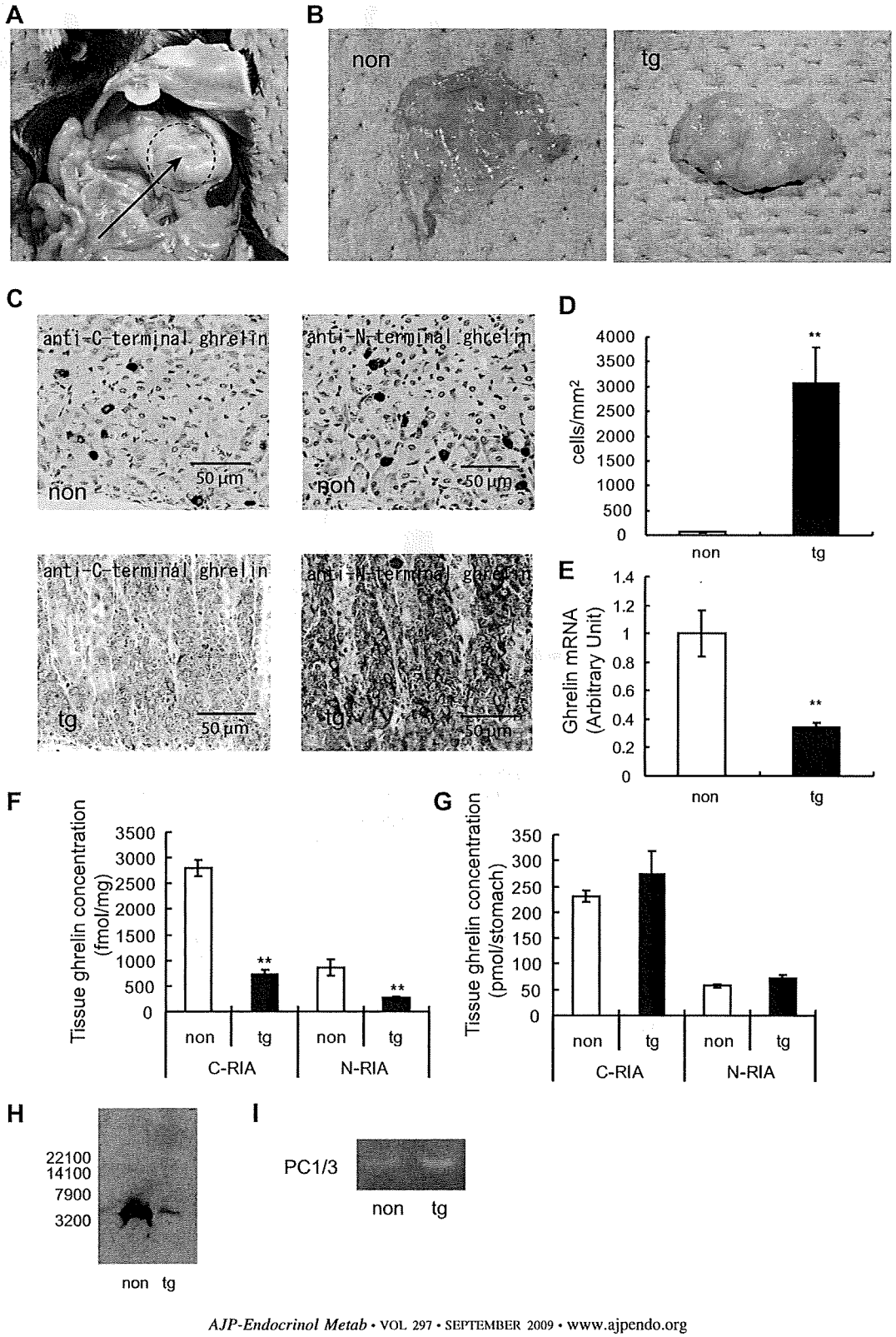
Generation of GP-Tag Tg mice. By injecting transgenes into 846 eggs, we obtained 11 lines of GP (4.85) Tag Tg mouse. We succeeded in breeding three of these lines (1-5, 3-1, and 4-3). Among these three lines, mice of the 3-1 line developed gastric tumor and showed elevated plasma ghrelin levels, as described below. Mice of the 1-5 line showed very aggressive tumor development and died at ~13 wk of age because of thyroid, pancreatic, and gastric tumors. Mice of the 4-3 line showed very slow tumor development. The proliferation of ghrelin cells was

Fig. 2. Pathological findings and tissue ghrelin concentrations of stomachs in GP-Tag Tg mice. *A–C*: macro findings of stomachs in GP-Tag Tg mice (*A*: arrow, dotted area; *B*: Tg) and nontransgenic littermates (non; *B*) at 12 wk of age. Stomach walls of GP-Tag Tg mice were hypertrophic. *C*: immunohistochemical analysis of ghrelin peptide expression in tissue sections of stomachs of GP-Tag Tg mice (Tg) and nontransgenic littermates (non) using anti-COOH-terminal and anti-NH_2-terminal ghrelin antibodies. *D*: the cell number of ghrelin-immunopositive cells in Tg and non littermates. *E*: the mRNA levels of ghrelin in 12-wk-old male Tg mice and non littermates; $n = 5$, $^{**}P < 0.01$ compared with nontransgenic littermates. *F* and *G*: tissue concentration per milligram (*F*) and per stomach (*G*) of ghrelin peptide in 12-wk-old male Tg mice (black bars) and non littermates (open bars); $n = 6$, $^{**}P < 0.01$ compared with non littermates. C-RIA, total ghrelin (ghrelin and desacyl ghrelin); N-RIA, ghrelin. *H*: Western blot analysis of stomach samples of Tg and non littermates using anti COOH-terminal ghrelin antibody. *I*: RT-PCR analysis of prohormone convertase 1/3 (PC1/3) mRNA expression in the stomach of Tg.

modest even at 50 wk of age in the 4-3 line. Accordingly, we analyzed mainly GP-Tag Tg mice of the 3-1 line.

We could not get a transgene-positive mouse of GP (1479) Tag Tg mouse by injecting transgenes into 631 eggs.

The expression levels of SV40-Tag mRNA among various tissues. We first examined the expression levels of SV40-Tag mRNA in various tissues of GP-Tag Tg mice, including stomach, small intestine, colon, hypothalamus, pituitary, thyroid,



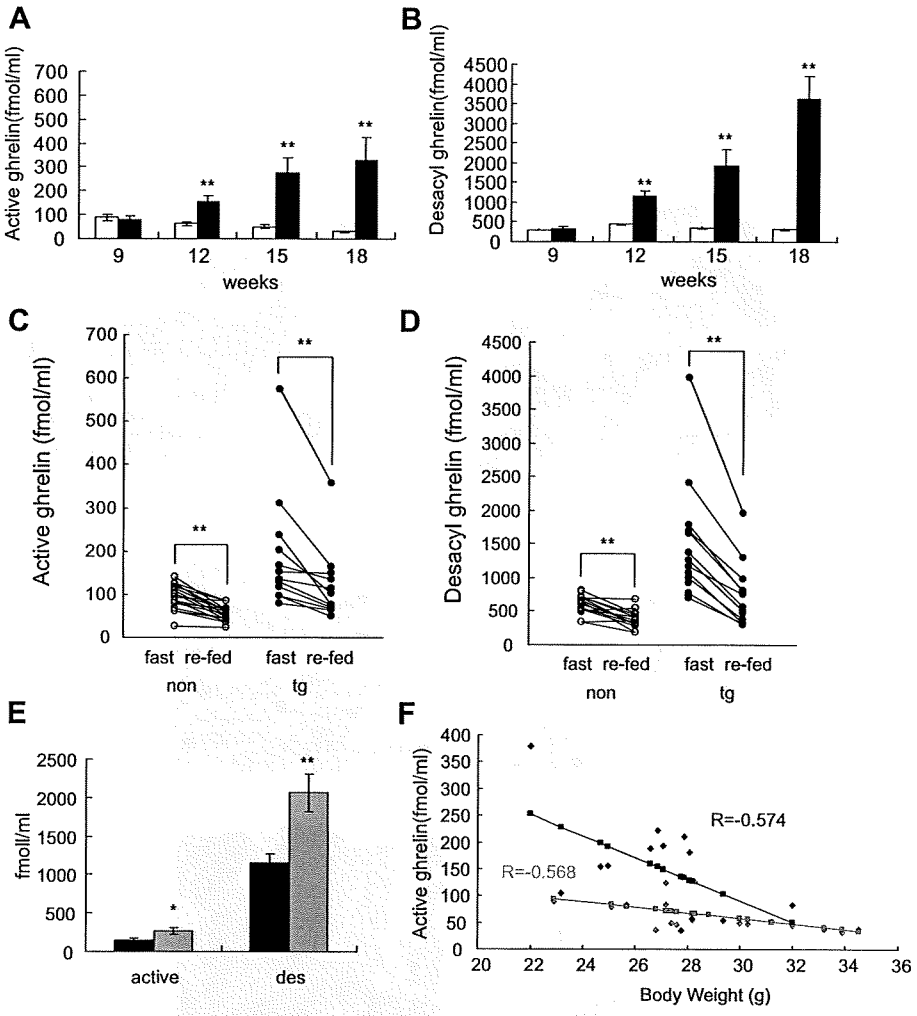


Fig. 3. Plasma ghrelin and desacyl ghrelin levels in GP-Tag Tg mice. *A* and *B*: plasma ghrelin (*A*) and desacyl ghrelin (*B*) levels in male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 3$–17. $^{**}P < 0.01$ compared with non littermates. *C* and *D*: plasma ghrelin (*C*) and desacyl ghrelin (*D*) levels after overnight fasting (fast) and after refeeding (refed) in 15-wk-old male Tg and non mice. $^{**}P < 0.01$; $n = 12$–18. *E*: plasma ghrelin (active) and desacyl ghrelin (des) levels in 12-wk-old male (black bars) and female (gray bars) GP-Tag Tg mice; $n = 7$–13, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with male GP-Tag Tg mice. *F*: plasma ghrelin levels were correlated with body weights in 12-wk-old male GP-Tag Tg mice (black bars; $r = -0.574$, $P < 0.01$) and in nontransgenic littermates (gray bars; $r = -0.568$, $P < 0.05$). The regression coefficient of the regression line of GP-Tag Tg mice was bigger than that of nontransgenic littermates ($t = 2.08$, $P < 0.05$).

pancreas, liver, heart, kidney, and testis (Fig. 1*B*). The highest expression levels were observed in stomach, and the second-highest levels were observed in small intestine. The expression pattern of SV40-Tag mRNA was almost similar to that of ghrelin (Fig. 1*C*).

Pathological feature and tissue ghrelin concentration of stomach of GP-Tag Tg mice. Stomach walls of GP-Tag Tg mice became hypertrophic with age (Fig. 2, *A* and *B*). Immunohistochemical analysis by both anti-COOH-terminal and anti-NH_2-terminal ghrelin antibodies revealed hyperplasia of ghrelin-immunopositive cells (Fig. 2, *C* and *D*), although the staining in GP-Tag Tg mice was paler than that in nontransgenic littermates (Fig. 2*C*). These hyperproliferating cells were not immunostained with anti-glucagon, somatostatin, or gastrin antibodies (data not shown).

The mRNA levels of ghrelin in the stomachs of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates ($P < 0.01$, $n = 6$; Fig. 2*E*). Consistent with this observation, tissue concentrations of ghrelin (N-RIA; fmol/mg tissue) and total ghrelin (desacyl ghrelin plus ghrelin) (C-RIA) of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates ($P < 0.01$, $n = 6$; Fig. 2*F*). However, since the weights of the stomach of GP-Tag Tg mice were significantly higher than controls (non-Tg vs. Tg, 83.4 vs. 362.0 mg, $P < 0.01$) due to the hypertrophy of the stomach wall, the tissue ghrelin concentration per whole stomach tended to be higher in GP-Tag Tg mice [not significant (NS), $n = 6$; Fig. 2*G*]. The size of ghrelin content of GP-Tag Tg mice was similar to that of nontransgenic littermates when analyzed by tricine-SDS PAGE and Western blot analysis (Fig. 2*H*), indicating that processing of preproghrelin to ghrelin occurred in hyperproliferaing ghrelin cells in GP-Tag Tg mice. The mRNA of prohormone convertase 1/3, which processes preproghrelin to ghrelin, was detected in the stomachs of GP-Tag Tg mice (Fig. 2*I*).

Plasma ghrelin levels of GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were almost equal to those of nontransgenic littermates at 9 wk of age and then increased with age ($n = 3$–17; Fig. 3, *A* and *B*), with some variations in the levels among animals.

We next examined whether physiological regulation of ghrelin secretion is preserved in GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were increased by fasting and decreased by refeeding $P < 0.01$, ($n = 7$–13; Fig. 3, *C* and *D*). Plasma ghrelin and desacyl ghrelin levels of female GP-Tag Tg mice were significantly higher than those of

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

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

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

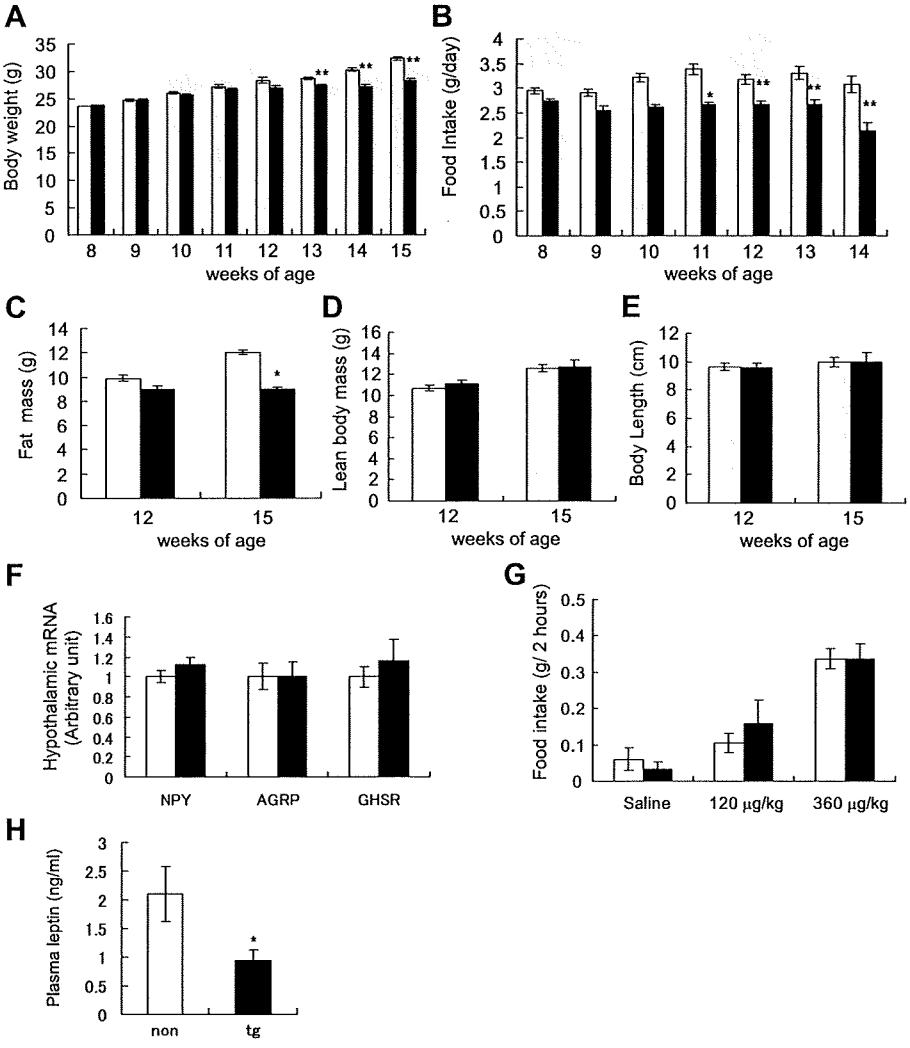


Fig. 4. Body weights, body compositions, and food intakes of GP-Tag Tg mice. *A*: body weights of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 22$–34. *B*: daily food intakes of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 19$–26.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