1. Introduction

Diabetic hyperphagia has the potential to hinder the success of following dietary advice. A modest number of patients with type 2 diabetes mellitus, from 5% to 21%, are estimated to have an abnormal eating behavior [1,2]. However, to date, the mechanisms relating how and the reason why hyperphagia is relevant to patients with diabetes mellitus have not been fully elucidated.

Ghrelin, originally identified as a growth hormone secretagogue, is an orexigenic gut hormone. It is a 28-amino-acid peptide produced by the X/A-like endocrine cells in the oxyntic glands of the gastric fundus [3,4]. The biological activities of ghrelin require octanoylation of the peptide on Ser3, an unusual posttranslational modification that is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT) [5,6].

Increased ghrelin signaling has been shown to contribute to the pathogenesis of diabetic hyperphagia [7,8] using streptozotocin-induced diabetic rat models with an observation period of 14 days or less, presumably due to the difficulty of maintaining the animals in the absence of insulin treatment. As central and peripheral administration of insulin in rats is known to suppress food intake and decrease neuropeptide Y messenger RNA (mRNA) [9], a model using treatment with insulin injection is not appropriate for investigating the appetite and eating disorders in the diabetic state.

However, a new inbred strain, the spontaneously diabetic Torii (SDT) rat, survives for extended periods of time characterized by hyperglycemia and hypoinsulinemia in the absence of ketonuria without insulin treatment [10] and thus can serve as a model of nonobese type 2 diabetes mellitus. As no exploratory study has been performed to date for the long-term involvement of ghrelin in eating behavior, we investigated this mechanism using SDT rats in the present study.

2. Methods

2.1. *Animals and protocols*

Male SDT rats (Japan SLC, Tokyo, Japan) and age-matched male Sprague-Dawley (SD) rats, used as controls, were housed in a controlled room under light conditions (lights on from 7:00 AM to 7:00 PM) with ad libitum access to standard laboratory chow and water from 8 to 35 weeks of age. The body weight and daily food intake of the rats were measured once a week throughout study period. The SDT and SD (n = 8, respectively) rats were euthanized at 8, 25, and 38 weeks of age; and blood and whole stomach samples were obtained. All experiments were conducted in accordance with the Regulation for Animal Experimentation in Kurume University.

2.2. *Blood sampling and biochemical analysis*

Blood samples were obtained via heart puncture into fluoride tubes for blood glucose, EDTA-2Na tubes for insulin and leptin measurement, and EDTA-2Na tubes with aprotinin for ghrelin analysis. After centrifugation (3500 rpm for 15 minutes at 4°C), plasma glucose was measured using a standard method; and the plasma insulin and leptin concentrations were measured using an enzyme-linked immunosorbent assay with commercially available kits (Morinaga Biochemical Research Laboratory, Yokohama, Japan).

2.3. *Radioimmunoassay for ghrelin*

Plasma samples for ghrelin were prepared by adding HCl (final concentration of 0.1 N) followed by extraction using a Sep-Pak C18 cartridge (Waters, Milford, MA) [4,11]. The eluate was lyophilized and stored at −80°C until the assay was performed. Stomach samples were extracted following homogenization. The supernatants were centrifuged for 15 minutes at 15 000 rpm, lyophilized, and stored at −80°C until the assay was performed.

Lyophilized samples were dissolved in radioimmunoassay (RIA) buffer on the day of assay. Ghrelin RIAs were performed as previously described [12,13] using 2 rabbit antisera against the N-terminal (Gly1-Lys11 with O-n-octanoylation at Ser3) or C-terminal (Gln13-Arg28) fragments of rat ghrelin to determine either the active form or total amount of peptide.

2.4. *Immunohistochemistry of ghrelin-immunopositive cells in the stomach*

The stomach samples were prepared for immunohistochemical analysis as described previously [14] using a rabbit anti-O-ghrelin antiserum (the same antiserum used for the ghrelin RIA as described above). The number of ghrelin-immunopositive cells (ip-ghrelin cells) per unit area of mucosal area (square millimeter) was counted using the Meta Morph software system (Molecular Devices, Tokyo, Japan).

2.5. *Reverse transcriptase polymerase chain reaction for mRNAs of ghrelin and GOAT*

The expression levels of mRNA for ghrelin and GOAT in stomachs of rats were examined using semiquantitative reverse transcriptase polymerase chain reaction (PCR) as described previously [15]. The PCR was performed using a commercially available PCR kit (Go-Taq Master Mix; Promega, Madison, WI, USA) with each primer set necessary to amplify the transcripts for ghrelin (accession no. NM_021669, 32 cycles) and GOAT (accession no. NM_001107317, 36 cycles). The National Institutes of Health Image (http://rsb.info.nih.gov/nih-image">http://rsb.info.nih.gov/nih-image">http://rsb.info.nih.gov/nih-image) program was used to determine the relative amount of each PCR product after gel electrophoresis, and the amount was normalized using the simultaneously amplified β-actin.

2.6. *Statistical analysis*

All tests were performed using SAS version 9.2 (SAS Institute, Cary, NC). Data are presented as the mean ± SD. Two-way analysis of variance followed with a post hoc test (Dunnett test) was used for the comparisons of the data obtained from 3

groups of rats with different ages. For the comparisons of the data between SD and SDT rats of the same age, Student unpaired *t* test was used for parametric data; or Mann-Whitney *U* test was used for nonparametric data. A *P* value < .05 was considered to be statistically significant.

3. Results

3.1. Body weight, daily food intake

The body weight of the SDT rats was significantly lower than that of the SD rats, despite a significantly larger amount of food intake, at both 25 and 38 weeks of age. Furthermore, in SDT rats, the daily food consumption at 38 weeks of age exceeded that at 25 weeks of age (Table 1).

3.2. Plasma concentrations of glucose, insulin, and leptin

Nonfasting plasma glucose concentrations of SDT rats at both 25 and 38 weeks of age were significantly higher than those of SD rats of the same age, respectively. At both 25 and 38 weeks of age, the plasma insulin and leptin concentrations of SDT rats were significantly lower than at 8 weeks of age and significantly lower than those of SD rats of the same age, respectively (Table 1).

3.3. Plasma and stomach levels of active ghrelin, total ghrelin, and the ratio of active ghrelin to total ghrelin

The plasma active ghrelin levels of SDT rats were significantly higher than those of SD rats at 38 weeks of age, whereas plasma total ghrelin levels in SDT rats were significantly lower compared with those of SD rats at 25 weeks of age. Consequently, at 38 weeks of age, the plasma ratios of active ghrelin to total ghrelin levels (A/T ratios) in SDT rats were significantly higher than those of SD rats.

Both active and total ghrelin contents in the stomach of SDT rats were significantly lower than those of SD rats at both 25 and 38 weeks of age. However, the stomach A/T ratios of SDT rats were significantly higher compared with those of SD rats at 25 and 38 weeks of age (Fig. 1).

3.4. Distribution of ghrelin-producing cells in the stomach

Ghrelin-immunopositive cells in the stomachs of both SD and SDT rats at 8 and 38 weeks of age were sparsely distributed in the lower part of the gastric mucosal layer, where they were moderately abundant. At 38 weeks of age, the number of ip-ghrelin cells per unit area of the stomach mucosa in SDT rats was significantly lower than that in SD rats (Fig. 1).

3.5. Stomach expression levels of ghrelin and GOAT mRNA

The relative mRNA levels of ghrelin in the stomach, corrected to β-actin levels, in SDT rats were significantly ($P < .05$) higher at both 25 and 38 weeks of age (1.17 ± 0.12 and 1.81 ± 0.31, respectively) compared with those in SD rats at the same ages (1.02 ± 0.04 and 1.38 ± 0.20, respectively). Concurrently, the GOAT mRNA level was significantly ($P < .05$) higher in SDT rats at 38 weeks of age (1.96 ± 0.23) than in SD rats (1.50 ± 0.26). The expression levels of these 2 transcripts were not significantly different between SD and SDT rats at 8 weeks of age.

4. Discussion

The major findings of the present study are described below. First, after the onset of diabetes, SDT rats exhibited a significant weight loss despite exaggerated food consumption. Second, SDT rats showed overt hyperglycemia at 25 and 38 weeks of age with a concomitant hypoinsulinemia and hypoleptinemia. Third, the plasma active ghrelin levels and ghrelin A/T ratio of SDT rats increased after the induction of diabetes, which was correlated with an increase of stomach ghrelin A/T ratio and ghrelin and GOAT mRNA expression levels.

Interestingly, stomach ghrelin contents and ip-ghrelin cell numbers were decreased in SDT rats compared with control SD rats, suggesting that active ghrelin was produced de novo in the stomach. Nevertheless, the increased A/T ratio of stomach ghrelin content in SDT rats was indicative of an active ghrelin

Table 1 – Comparisons of parameters between SD and SDT rats at each age

	Age (wk)	Body weight (g)	Food intake (g/d)	Glucose (mmol/L)	Insulin (pmol/L)	Leptin (ng/mL)
SD	8	240.0 ± 7.1 (231.2-248.8)	27.6 ± 2.1 (25.0-30.2)	9.65 ± 0.56 (9.06-10.22)	359.1 ± 123.6 (167.8-720.6)	1.75 ± 0.45 (1.16-2.29)
	25	642.5 ± 26.6 * (620.3-664.7)	29.5 ± 2.2 (26.9-32.2)	9.32 ± 0.91 (8.56-10.07)	991.6 ± 182.4 * (469.5-1226.4)	5.81 ± 1.25 * (4.50-7.13)
	38	619.0 ± 66.4 * (549.0-689.0)	28.4 ± 1.5 (26.6-30.2)	9.61 ± 0.31 (9.28-9.93)	879.8 ± 166.2 * (671.8-1083.6)	5.29 ± 1.65 * (3.58-7.60)
SDT	8	302.1 ± 39.8 (265.4-338.9)	29.9 ± 1.2 (28.7-31.1)	9.04 ± 0.53 (8.57-9.69)	340.1 ± 78.8 (68.1-610.7)	2.20 ± 0.51 (1.67-2.73)
	25	454.3 ± 41.6 †,‖ (415.8-492.7)	45.5 ± 5.8 †,‖ (38.4-52.7)	28.45 ± 2.07 †,‖ (26.51-30.33)	73.1 ± 19.3 ‡,‖ (52.8-93.1)	0.94 ± 0.26 ‡,‖ (0.67-1.22)
	38	414.2 ± 10.0 †,‖ (403.8-424.5)	55.8 ± 5.9 †,§,‖ (48.4-63.1)	30.02 ± 1.26 †,‖ (28.68-31.32)	34.1 ± 1.4 ‡,‖ (31.9-36.3)	0.37 ± 0.08 ‡,‖ (0.28-0.46)

Values are presented as means ± SD. Ninety-five percent confidence intervals are shown in parentheses.

* $P < .001$ vs 8-week-old SD rats.

† $P < .001$ vs 8-week-old SDT rats.

‡ $P < .05$ vs 8-week-old SDT rats.

§ $P < .05$ vs 25-week-old SDT rats.

‖ $P < .001$ vs SD rats at the same age.

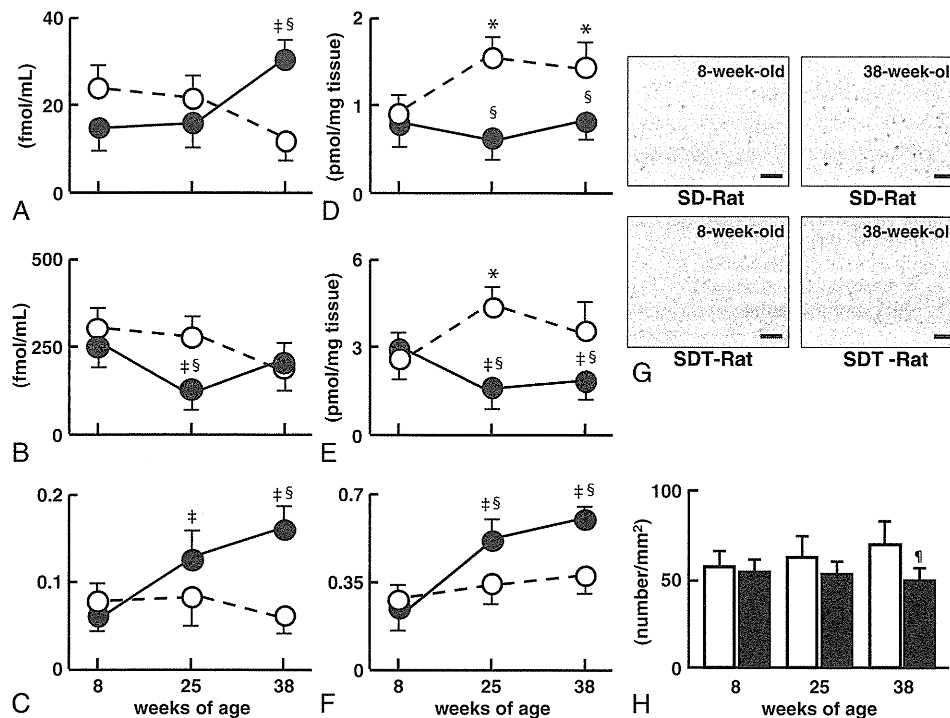

Fig. 1 – Plasma and stomach ghrelin contents and the localization of ip-ghrelin cells in the stomach mucosa of SD or SDT rats at different ages. A to C, Plasma ghrelin concentrations. D to F, Stomach ghrelin contents. A and B,: Active ghrelin. C and D, Total ghrelin. E and F, Ratio of active ghrelin to total ghrelin. G, Immunohistochemistry of ip-ghrelin cells (scale bar = 100 μm). H, The number of ip-ghrelin cells (the means of random 20 sections from each rat). White circles with dotted lines and white bars indicate SD rats, and black circles with solid lines and black bars indicate SDT rats. Data are presented as the mean ± SD. *P < .01 vs values from 8-week-old SD rats. †P < .05 vs values from 8-week-old SDT rats. ‡P < .01 vs 8-week-old SDT rats. §P < .01 vs SD rats at the same age. ¶P < .001 vs SD rats at the same age.

production to meet a demand, which was further confirmed by higher levels of ghrelin and GOAT mRNA in stomach of SDT rats after the induction of diabetes. The increased ghrelin production in this diabetic model may be a compensatory mechanism to keep weight stable under conditions of diabetic malnourishment. Future experiments involving ghrelin antagonists may be helpful to explore this hypothesis.

The hyperphagia observed in SDT rats appears to arise, at least in part, from deficient hypothalamic signaling by insulin and leptin, which in turn leads to the activation of neuropeptide Y/AgRP (agouti-related protein) neurons [9,16] separate from the participation of the ghrelin production. Furthermore, reciprocal relationships exist between ghrelin and insulin and between ghrelin and leptin in plasma concentration [17]. Notably, the peripheral administration of insulin to diabetic women suppressed plasma ghrelin concentrations [18]. Whereas the mechanisms involved in ghrelin production in SDT rats are largely unknown, suppressed insulin or leptin levels could be one of the possible mechanisms.

In conclusion, SDT rats with uncontrolled hyperglycemia may serve as a model of hyperphagia often observed in nonobese patients with type 2 diabetes mellitus. Active ghrelin production is concerned with diabetic hyperphagia solely or in concert with the suppression of insulin or leptin. This report elucidated the relevance of ghrelin to diabetic hyperphagia in a diabetic state over an extended period.

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Conflict of Interest

The authors declare that there is no conflict of interest associated with this manuscript.

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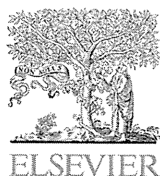

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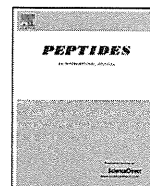

Preface

The discovery of ghrelin: With a little luck and great passion

To discover a novel peptide hormone needs a little luck and hard work. When we discovered ghrelin, we were young and enthusiastic about peptide hunting. If one of our team members, Kenji Kangawa, Hiroshi Hosoda, or I, were not involved, ghrelin would not have been discovered.

Kenji and I moved from Miyazaki Medical College to the National Cardiovascular Center Research Institute in Osaka in 1993 and began to search for novel peptide hormones. However, we could not find any novel peptides for the first six years, except for known peptides and fragments derived from non-peptide hormone proteins. We decided to change the target receptors and picked up the GHS-R, now called the ghrelin receptor.

Hiroshi joined us as a graduate student to take a PhD degree just before we had tackled the search for the endogenous ligand to the GHS-R. Hiroshi was a surgeon and had no experience in basic medical research. He was bewildered by the gambling approach of peptide hunting and thought research to find a novel peptide was risky. In general most scientists would be discouraged by everyday negative results; however, Hiroshi had a lot of guts and was optimistic.

By January 1999, almost a year after beginning our hunt for the GHS-R ligand, we still had not come up with any leads. We had labored step by step with our chromatography and completed more than 500 assays without any hint of the ligand. We began to toy with the notion that we should change the target tissue from brain to other tissues. But this required courage: we, and perhaps others, had assumed from the distribution pattern of the GHS-R that brain tissue was the probable production site for the endogenous ligand.

Unexpectedly, the stomach contained excessive levels of the endogenous ligand. However, it was lucky that we used fresh extracted samples of stomach, because ghrelin is inactivated during long storage in acid. Only several mg of the stomach extract was sufficient for detecting the activity. With great surprise we had discovered a novel peptide hormone in the stomach! Encouraged anew, we happily set about purifying the ligand and soon succeeded. On May 30, after a mere 10 days, the ligand was purified from just a few milligrams of stomach tissue. However, the structure of the peptide hormone was beyond all conception.

After purifying the GHS-R ligand, we worked harder than before. Amino acid sequence analysis of the purified ligand revealed no signal from the third amino acid, whereas a cDNA analysis of the ligand identified the unknown third amino acid as a serine. What prevented us from detecting the third amino acid serine in the sequence analysis? When we obtained the synthetic non-acyl ligand a week later and checked the activity, amazingly there was none! We repeated the assay again and again, but the results were the same. The synthetic ligand had no activity! When we compared the purified and synthetic ligands we found that their elusion positions on HPLC were very different, indicating that they had different structure. We speculated that the serine residue at the third position might have been modified by an unknown molecule, and that the activity could not take place without such a modification. What was the modification?

I remember well a hot day in early June 1999. The day when n-octanoyl ghrelin was synthesized based on our proposed structure. At last we had the answer! The structure of ghrelin is unprecedented; ghrelin is a peptide hormone in which the serine 3 (Ser^3) is n-octanoylated and this modification is essential for ghrelin's activity. It was a tremendous thrill to have discovered a novel peptide, and one with a structure no one had ever imagined.

The discovery of ghrelin changed our life, and of course for the better. Now one of us (MK) runs his own lab at Kurume University. Yet still we wonder what would have happened if we had not changed the target tissue from brain to stomach, or if we had failed to solve the modified structure of ghrelin. Good results in research depend partly on luck. Though, it may be hackneyed to profess that good luck in research depends on the researcher's passion, I believe it is true.

Lastly, let me (MK) express my personal pleasure. I am very happy and honored to find that "ghrelin" is described in Lehninger's Biochemistry and Stryer's Biochemistry, which I studied as a text book in biochemical courses in my college student days. I could not have imagined when I was a young student that our discovery would be included in these text books many years later, . . . and that it would give rise to a Special Issue of PEPTIDES.

From left: Masayasu Kojima, Kenji Kangawa and Hiroshi Hosoda.

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Postoperative Weight Loss Does Not Resolve After Esophagectomy Despite Normal Serum Ghrelin Levels

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Background. Esophagectomy after gastric reconstruction leads to significant weight loss. Ghrelin is known to stimulate appetite and cause weight increase. The objective of this study is to examine the relationship of serum ghrelin levels and weight loss in patients after esophagectomy for cancer.

Methods. Twenty-two patients underwent esophagectomy including gastric reconstruction. Serum ghrelin levels and weight were measured preoperatively and then postoperatively for 12 months in all patients. A questionnaire assessed appetite, amount of food eaten, satisfaction, and frequency of eating.

Results. Preoperatively, the mean serum ghrelin level was 67.9 ± 42.6 (fmol/mL ± SD), and at 1, 3, 6, and 12 months after surgery were 43.4 ± 28.1, 51.5 ± 32.2, 67.1 ± 50.9, and 84.9 ± 43.1, respectively. Compared with preoperative values, the mean body mass index decreased by 1.9 ± 1.5, 2.3 ± 1.8, 2.1 ± 2.3, 2.4 ± 2.7 at 1, 3, 6, and 12 months after surgery. While appetite score showed a decrease at 1 month (1.6 ± 0.92), appetite increased by 12 months postoperatively (2.7 ± 1.0) and showed a strong positive correlation (r = 0.743) with serum ghrelin levels. There were no significant differences in ghrelin levels when patients were stratified by disease stage, recurrence, or administration of adjuvant chemotherapy.

Conclusions. Esophagectomy resulted in temporary reduction of ghrelin levels, but while levels returned to normal 3 months later, weight loss persisted at 12 months. Further study is needed to elucidate the mechanisms of persistent weight loss and design therapeutic interventions to recover the weight lost.

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Esophagectomy with gastric replacement may lead to significant weight loss and cause a variety of symptoms including dysphagia, gastroesophageal reflux, aspiration, choking, dyspnea, cough, and hoarseness [1–6]. Weight loss is a reliable indicator of postoperative malnutrition and profoundly affects overall postoperative quality of life [7, 8]. Significant weight loss after esophagectomy has been reported to be about 10% to 20% of preoperative weight [4, 5], similar to that after total gastrectomy [9, 10].

Ghrelin was first identified as a ligand for the growth hormone secretagogue receptor of the pituitary gland in 1999 [11]. Recent studies reveal that ghrelin stimulates not only growth hormone secretion from the pituitary gland but also the appetite signal in the hypothalamus, in opposition to leptin [12], an appetite-suppressing hormone [13]. Most ghrelin is secreted by the stomach, with only trace amounts from other organs [14]. The discovery of ghrelin led to the novel concept of weight regulation by the stomach, which has been investigated in bariatric surgery and in patients after gastrectomy [15–17]. There is a single study of ghrelin levels after esophagectomy with gastric tube reconstruction, despite the fact that this procedure including anatomic translocation of the stomach and vagotomy may affect ghrelin production from the stomach [18]. This is a prospective study to examine the long-term relationship between serum ghrelin levels and weight loss in a single cohort of patients with thoracic esophageal squamous cell carcinoma after esophagectomy.

Patients and Methods

Patients

This study was approved by the Institutional Review Board of Jichi Medical University, and individual consent obtained. Esophageal cancer patients who underwent resection at Jichi Medical University Hospital from 2004 through 2005 were enrolled. Inclusion criteria included

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the following: (1) thoracic esophageal carcinoma; (2) reconstruction with a gastric tube; (3) no serious postoperative complication; (4) no other gastrointestinal diseases; (5) no serious dysfunction [white blood cell count 4,000 to 12,000/mm^3; platelet count $\geq$ 100,000 /mm^3; hemoglobin $\geq$ 8.0 g/dL; total bilirubin $\leq$ 1.5 mg/dL, glutamic-oxaloacetic acid transaminase $\leq$ 80 U/L; glutamic-pyruvic acid transaminase $\leq$ 80 U/L; alkaline phosphatase $\leq$ 2 times normal; creatinine $\leq$ 1.5 mg/dL]; and (6) informed consent.

Surgical Procedure

All patients underwent a right-sided thoracotomy and esophagectomy with lymph node dissection. A gastric tube was created along the greater curvature, approximately 5 cm in diameter. The finger bougie method to assure drainage of the vagotomized posterior mediastinal stomach was used. Reconstruction was performed using a retromediastinal and cervical anastomosis, or an intrathoracic anastomosis. Bilateral truncal vagotomy was performed distal to the bronchial branch. Jejunostomy tubes were not placed in any of the patients. Patients with lymph node metastases (stage III-IV) received adjuvant chemotherapy using two courses of 5-fluorouracil and cisplatin. Patients received individualized counseling and guidance regarding diet and eating habits in the postoperative period.

Table 1. Postoperative Questionnaire

(1) How does your appetite now compare with your appetite before surgery?	
Decreased	1 (points)
Decreased slightly	2
Same as before	3
Increased slightly	4
Increased	5
(2) How much do you eat now, compared with before surgery?	
1/4 or less	1 (points)
1/4 to 1/2	2
1/2 to 3/4	3
3/4 to same	4
Increased	5
(3) How many times do you eat each day?	
1 time	1
2 times	2
3 times	3
4 times	4
5 times	5
(4) How "full" do you feel after eating a meal now, compared with before surgery (eating satisfaction)?	
Hungry	1 (points)
Slightly hungry	2
Normal	3
Full (slightly)	4
Full	5

Table 2. Patient Demographic Information and Perioperative Condition

Total number of patients	22
Age (years), mean ± SD	61.5 ± 7.4
Range	45–80
Gender	
Male	20
Female	2
Operation	
Esophagectomy	22
TNM stage	
I	2
II	13
III	5
IV	2
Preoperative BMI	21.7 ± 7.3
Range	13.7–30.5
<20	7
20–25	12
>25	3
Preoperative comorbidities	
None	10
Hypertension	7
Anemia	2
Postmyocardial infarction	1
COPD	1
Chronic renal failure	1
Postcerebral hemorrhage	1
Hyperlipidemia	1
Benign prostatic Hypertrophy	1
Retinitis pigmentosa	1
Postoperative complications	5
Recurrent laryngeal nerve palsy	1
Wound infection	1
Liver dysfunction	1
Arrhythmia	1
Bronchial asthma	1

BMI = body mass index; COPD = chronic obstructive pulmonary disease; TNM = tumor-nodes-metastasis.

Measurement of Serum Ghrelin

Measurement of serum ghrelin was performed preoperatively and at 1, 3, 6, and 12 months, postoperatively. A 10 mL fasting blood sample was collected from each patient on the day of measurement.

Two rabbit polyclonal antisera were used including one against the COOH-terminal Cys-extended rat ghrelin, to recognize ghrelin with n-octanoylated Ser 3 (acylated ghrelin; ghrelin). Radioimmunoassay (RIA) using this antiserum, designated NH_2-terminal radioimmunoassay, determined the concentration of acylated ghrelin. The other antiserum was raised against the NH_2-terminal Cys-extended rat ghrelin to recognize both acylated ghrelin and desacyl-ghrelin [19]. Radioimmunoassay using this antiserum, COOH-terminal radioimmunoassay, determined the concentration of acylated ghrelin plus desacyl-ghrelin. "Acylated ghrelin" is described as

"ghrelin" and "acylated ghrelin plus desacyl-ghrelin" is described as "total ghrelin" in this study.

Questionnaire

Appetite, amount of food eaten, and satisfaction after eating were scored from 1 to 5 subjectively, comparing the patient's condition to their preoperative status (Table 1). Patients were asked how many times each day they ate. Weight (kg) and body mass index (BMI; kg/m^2) were measured preoperatively and at 1, 3, 6, and 12 months postoperatively.

Statistical Analysis

Statistical analysis was performed with the SPSS statistical package, version 14 (SPSS, Chicago, IL). Data are expressed as mean ± SD. Differences in each study variable were compared using one-way analysis of variance. A p value less than 0.05 was considered statistically significant.

Results

Twenty-two patients underwent esophagectomy, including 20 males and 2 females. The average age was 65.0 years (range 45 to 84). Patient demographic information is shown in Table 2.

Serum Ghrelin Levels

The mean concentration of serum total ghrelin and serum ghrelin are shown in Table 3. There is a significant ($p < 0.05$) reduction at one month for both total ghrelin and ghrelin compared with the preoperative value. Total ghrelin and serum ghrelin levels at 12 months are significantly higher than at 1 month postoperatively, demonstrating a recovery of ghrelin level by 12 months after resection.

Body Mass Index and Ghrelin Levels

Preoperative BMI is shown in Table 2. Compared with preoperative BMI, the decrease in BMI was 1.9 ± 1.5, 2.3 ± 1.8, 2.1 ± 2.3, and 2.4 ± 2.7 at 1, 3, 6, and 12 months after surgery, respectively, as shown in Table 3. These changes are all significantly lower than preoperative levels, but not significantly different at each time point documenting a persistent loss of weight. There was no correlation between preoperative BMI and serum ghrelin level. There was no correlation between the decrease in BMI and the serum ghrelin level postoperatively (correlation coefficient = 0.01).

Serum Albumin Level

Serum albumin levels were measured preoperatively and at 1, 3, 6, and 12 months, and are shown in Table 3. Serum albumin was significantly lower at one month compared to the preoperative level. Serum albumin levels showed a significant increase at 6 months postoperatively, compared with the level at one month postoperatively.

Pathologic Stage and Serum Ghrelin Levels

The tumor-nodes-metastasis stage, using the Japanese Esophageal Society version 5, showed the following: stage I, n = 2; stage II, 13; stage III, 5; and stage IV, 2. The serum concentration of total ghrelin showed no significant difference among any of the tumor stages at any time point, as shown in Table 4.

Adjuvant Chemotherapy and Ghrelin Levels

The serum concentration of total ghrelin was 73.1 ± 35.2 (fmol/mL ± SD) in those who received adjuvant chemotherapy (n = 7) (AC+) and 64.3 ± 48.1 (fmol/mL ± SD) in patients who did not (n = 15) (AC−), preoperatively. Serum total ghrelin levels stratified by the use of adjuvant chemotherapy is shown in Table 4, and there are no significant differences between the groups.

Tumor Recurrence and Ghrelin Levels

In patients with recurrent disease (n = 3), the concentration of total ghrelin was 78.5 ± 20.3 (fmol/mL ± SD) and 66.2 ± 45.2 (fmol/mL ± SD) in patients without recurrence (n = 19), preoperatively. The serum levels of total ghrelin stratified by recurrence are shown in Table 4 and there are no significant differences between the two groups.

Table 3. Total Ghrelin, Ghrelin, Change in Body Mass Index (BMI) and Serum Albumin

Parameter	Preoperative	1 month	3 months	6 months	12 months
Total Ghrelin (fmol/mL)	67.9 ± 42.6	43.4 ± 28.1[a] (p = 0.007)	51.5 ± 32.2	67.1 ± 50.9	84.9 ± 43.1[b] (p = 0.008)
Ghrelin (fmol/mL)	9.0 ± 8.3	5.3 ± 4.3[a] (p = 0.024)	6.5 ± 5.2	7.6 ± 4.6	10.5 ± 6.7[b] (p = 0.03)
ΔBMI	0	1.9 ± 1.5[a] (p = 0.016)	2.3 ± 1.8[a] (p = 0.001)	2.1 ± 2.3[a] (p = 0.004)	2.4 ± 2.7[a] (p = 0.001)
Albumin (mg/dL)	4.0 ± 0.5	3.7 ± 0.3[a] (p = 0.011)	4.0 ± 0.3	4.0 ± 0.3[b] (p = 0.023)	4.0 ± 0.4

[a] Result is significantly different compared with preoperative value, by ANOVA analysis. [b] Result is significantly different compared with that at 1 month, by ANOVA analysis.

ANOVA = analysis of variance.

Table 4. Serum Total Ghrelin Level (fmol/mL) Stratified by Clinical Course

Clinical Parameter	Preoperative	1 month	3 months	6 months	12 months
Pathologic stage					
I	25.5 ± 3.0	18.5 ± 4.7	20.2 ± 1.6	29.2 ± 6.6	54.9 ± 9.7
II	72.2 ± 44.4	48.3 ± 29.1	57.9 ± 34.4	79.0 ± 60.8	93.3 ± 48.2
III	64.7 ± 48.7	48.9 ± 30.9	43.8 ± 26.5	45.4 ± 21.5	63.3 ± 25.5
IV	89.9 ± 7.3	22.7 ± 2.1	60.4 ± 41.9	81.4 ± 26.4	114.1 ± 41.0
Adjuvant chemotherapy					
Yes	73.1 ± 35.2	44.2 ± 25.1	46.9 ± 27.1	48.7 ± 26.0	82.4 ± 40.0
No	64.3 ± 48.1	42.9 ± 31.0	54.7 ± 36.0	79.8 ± 60.5	86.7 ± 46.7
Recurrent disease					
Yes	78.5 ± 20.3	32.9 ± 17.6	67.9 ± 32.4	78.4 ± 19.4	107.5 ± 31.2
No	66.2 ± 45.2	45.1 ± 29.4	48.9 ± 32.2	65.2 ± 54.4	81.2 ± 44.3

There were no significant differences in total ghrelin levels in any of the groups at any of the time points as analyzed by one-way analysis of variance.

Questionnaire

Appetite scores were based on a questionnaire (Table 1). The average appetite score (question 1) was 1.62 ± 0.92 at 1 month; it increased gradually to 2.29 ± 1.15 at 3 months, 2.73 ± 1.42 at 6 months, and 2.73 ± 1.03 at 12 months postoperatively. Scores are shown in Table 5, and changed significantly comparing the results at one month with those at six and 12 months after surgery ($p < 0.05$).

The amount consumed at each meal (question 2) decreased during the first month, but recovered to normal after that. There was a decrease in the frequency of meals (question 3), but it returned to the normal range after surgery (Table 5). There were significant changes at one and six months postoperatively. There were no significant changes in the sense of satisfaction score (question 4) after surgery (Table 5). Both appetite and ghrelin recovered over time, and there was a strong positive correlation (R = 0.862) (Fig 1).

Comment

Ghrelin, secreted by the fundic glands of the stomach, increases weight by stimulating appetite and growth hormone secretion [11, 14], in a negative feedback loop in relation to weight. After surgery that affects gastric function, reduction of ghrelin levels may be related to postoperative weight loss. Gastric bypass surgery results in both weight loss and a reduction in serum ghrelin concentration, and probably appetite as well, while starvation reduces body weight but increases the ghrelin concentration with the sensation of hunger [15, 17]. Patients with gastric cancer typically have a reduction in serum ghrelin levels of 80% and a persistent loss of weight of about 20% after total gastrectomy [20, 21]. Resection of esophageal cancer includes resection of the upper part of the stomach, and gastric volume is reduced to create the gastric tube used for reconstruction. Vagotomy is also performed in the esophageal cancer operation. In theory, the concentration of serum ghrelin is reduced, and in fact it was reduced significantly after operation in this study. Ghrelin reduction after esophageal cancer surgery is likely a combination of the vagotomy and the partial of resection of the stomach. Significantly reduced serum ghrelin levels were seen at one month after surgery, but levels began to increase three months later. Subsequent increases in ghrelin levels may be due to the fact that the gastric fundus is still intact, in contrast to patients under-

Table 5. Patient Responses to Satisfaction Questionnaire

Question	1 Month	3 Months	6 Months	12 Months
Appetite	1.6 ± 0.9	2.3 ± 1.2	2.7 ± 1.4[a]	2.7 ± 1.0[a]
			$p = 0.011$	$p = 0.011$
Amount	2.5 ± 1.0	2.9 ± 0.9	3.1 ± 1.1	3.1 ± 0.9
Meal Frequency	4.4 ± 1.3	3.6 ± 1.0[a]	3.6 ± 0.9[a]	3.1 ± 0.6[a]
		$p = 0.036$	$p = 0.039$	$p < 0.001$
Satisfaction	3.3 ± 1.2	3.7 ± 1.0	3.9 ± 0.9	4.0 ± 1.1

[a] Shows a significantly different ($p < 0.05$) score compared with that at 1 month.

Responses on a 1 to 5 point scale for each question at the time points shown.

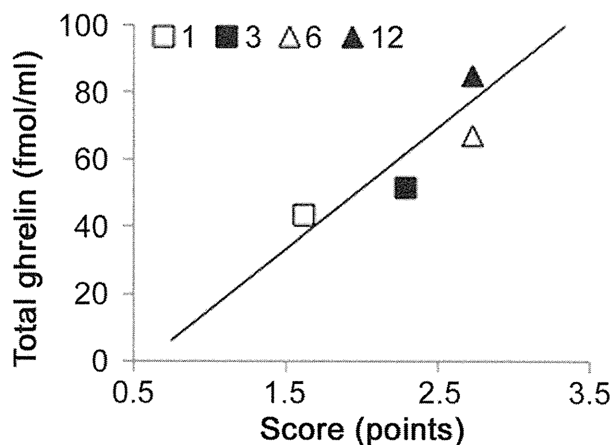

Fig 1. Correlation between total ghrelin level and appetite score. The correlation between total ghrelin and appetite score (points) was evaluated by a linear regression model, and shown by the regression line ($Y = 30.1X - 8.79$, $R^2 = 0.743$). (□ = 1; ■ =3; Δ = 6; ▲ = 12 months.)

going total gastrectomy and in whom ghrelin levels remain decreased at 12 months after resection (unpublished data). Performing a vagotomy may affect the neural influences on ghrelin production. The interaction between the vagus nerve and the fundus in regard to ghrelin production remain an area of active investigation. Transection of the vagus nerve has been reported to abolish the orexigenic effect of ghrelin in rodents [22, 23]. There are some reports that there is no relationship between ghrelin concentration and vagotomy in gastrectomy or esophagectomy [24, 25].

Doki and colleagues [18] reported the effect of esophagectomy on serum ghrelin and observed a correlation with ghrelin and postoperative body weight loss in esophageal cancer patients. They found a serum ghrelin concentration of 88.6 fmol/mL in the control group, which decreased to 58.8 fmol/mL 12 months postoperatively in the outpatient group. They concluded that the decline of ghrelin levels may play a role in weight loss after esophagectomy. The results of the present study are considerably different in that ghrelin levels returned to normal by 12 months postoperatively. One reason for the disparity of results may be that the data in the previous study were not analyzed continuously for the same group of patients. This is the only previous report about ghrelin and esophagectomy but the result is somewhat different from those in this prospective study in which all patients were followed continuously. This study was followed by a phase II clinical trial of ghrelin administration after esophagectomy [26], which showed a significantly increased food intake in patients receiving ghrelin compared with a placebo group, with attenuation of weight loss.

Weight loss is a significant issue for patients after esophagectomy. Clinical trials looking at operative variables such as the route of reconstruction or type of gastric tube (whole or narrow tube) did not improve calorie intake or limit postoperative weight loss [27, 28]. In this study, the BMI significantly decreased at all time points measured compared with preoperative values, and persisted through the entire study period despite recovery of serum ghrelin levels. A wide variation in energy expenditure may contribute to the results observed.

Responses to a questionnaire showed that appetite recovered chronologically, in parallel to the change in serum ghrelin levels. Serum ghrelin levels recovered early in the follow-up period, but did not synchronize with recovery of lost weight. About 70% of serum ghrelin is secreted from the stomach, so secretion may occur in the gastric tube during the postoperative period. There were no significant differences reported in the patients' satisfaction after eating.

Patients did report eating significantly more food at 12 months after surgery compared with the amount eaten at one month after surgery, correlating with increased ghrelin levels. This observation is consistent with the results of Yamamoto and colleagues [26], who found increased caloric intake in patients receiving exogenous ghrelin after esophagectomy. Further study will be needed to determine if a cause and effect relationship exists.

Ghrelin enhances appetite and increases food intake in humans. This study shows a strong correlation between total serum ghrelin levels and appetite score. Ghrelin increases energy intake in patients with impaired appetite [29]. le Roux and colleagues reported that ghrelin does not stimulate food intake in patients after upper intestinal surgical procedures involving vagotomy (3 total gastrectomy, 1 partial gastrectomy, and 3 esophagectomy patients did not alter their energy intake with an infusion of ghrelin) [30]. This double blind, placebo-controlled trial supports the conclusions of this study; that ghrelin may not play an important role in reduced weight loss after esophagectomy.

The stress of surgery alone could cause a change in ghrelin levels. In a study of patients after coronary artery bypass grafting, 17 patients were evaluated for ghrelin levels, including fasting and postprandial levels [31]. While fasting ghrelin levels were significantly increased postoperatively, postprandial ghrelin levels were suppressed.

Peripheral ghrelin administration leads an increase in food intake and growth hormone release in humans [32]. The safety and usefulness of recombinant ghrelin have been confirmed in clinical trials for patients with heart failure [33]. Based on the results of the present study, the role of exogenous ghrelin requires further investigation.

Weight loss may depend on other factors such as dysphagia, gastrointestinal reflux, aspiration, and diarrhea, which often accompany esophageal resection. Although serum ghrelin levels recover, patients do not recover weight lost postoperatively, suggesting that other factors are responsible for the persistent weight loss seen after esophageal resection. Future studies will include patient quality-of-life issues including gastrointestinal symptoms in searching for the factors responsible for weight loss, as well as other possible physiologic causes. Clinical trials using exogenous recombinant ghrelin therapy for patients after esophagectomy are necessary to more fully understand the complex physiology of weight loss after esophagectomy.

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INVITED COMMENTARY

Weight loss has been shown to be a significant predictor of survival in cancer patients. In patients with esophageal cancer undergoing esophagectomy, the weight loss is undoubtedly multifactorial, involving disturbed gastric emptying, gastroesophageal reflux, and depressed appetite as well as occasional swallowing dysfunction or anastomotic stricture. Recent interest has focused on the peptide hormone ghrelin and its role in enhancing eating and weight gain after gastroesophageal operations. Ghrelin, predominantly secreted by the stomach, has numerous biologic functions, including enhancing secretion of growth hormone, regulating energy balance, and promoting appetite. A randomized trial from Japan previously showed that supplementation of synthetic ghrelin postoperatively in patients after esophagectomy improved appetite and attenuated weight loss [1].

In the current study, Koizumi and colleagues [2] evaluated postoperative ghrelin levels after esophagectomy and related them to weight loss and appetite. They found that ghrelin levels decreased immediately after the operation, but returned to baseline levels by 3 months. Appetite scores steadily increased over the 12-month postoperative period as well. Body mass index, however, declined after the operation and failed to increase up to 1 year postoperatively. This was in a relatively lean patient cohort with a mean body mass index of only 21.7 kg/m^2.

Although interesting, the study still leaves many questions to be answered regarding the role of ghrelin in gastroesophageal operations. The exact cause of de-

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MINIREVIEW

Natriuretic peptide system: an overview of studies using genetically engineered animal models

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The mammalian natriuretic peptide system, consisting of at least three ligands and three receptors, plays critical roles in health and disease. Examination of genetically engineered animal models has suggested the significance of the natriuretic peptide system in cardiovascular, renal and skeletal homeostasis. The present review focuses on the *in vivo* roles of the natriuretic peptide system as demonstrated in transgenic and knockout animal models.

Natriuretic peptides

The existence of an atrial factor with diuretic and natriuretic activities has been postulated since 1981 [1]. In 1983–1984, the isolation and purification of such a factor and determination of its amino acid sequence were accomplished in rats and humans [2–7]. The factor is a peptide distributed mainly in the right and left cardiac atria within granules of myocytes and thus called atrial natriuretic factor or atrial natriuretic peptide (ANP). The discovery of ANP revealed that the heart is not only a mechanical pump driving the circulation of blood but also an endocrine organ regulating the cardiovascular–renal system. For instance, in situations of excessive fluid volume, cardiac ANP secretion is stimulated, which causes vasodilatation, increased renal glomerular filtration and salt/water excretion and inhibition of aldosterone release from the adrenal gland, which collectively result in a reduction of body fluid volume.

Later, in 1988, a homologous peptide with similar biological activities was isolated from porcine brain and hence was named brain natriuretic peptide (BNP) [8]. However, it was soon found that brain BNP levels were much lower in other species. It has since been shown that BNP is mainly produced and secreted by the heart ventricles [9]. Synthesis and secretion of BNP are regulated differently from ANP [10], and the plasma concentration of BNP has been found to reflect the severity of heart failure more closely than ANP [11].

In 1990, yet another type of natriuretic peptide was isolated from porcine brain and named C-type

Abbreviations
ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC, guanylyl cyclase; MCIP1, myocyte-enriched calcineurin-interacting protein; PAR, protease-activated receptor; PKG, cGMP-dependent protein kinase; RGS, regulator of G-protein signaling.

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natriuretic peptide (CNP) [12]. CNP was initially thought to function only in the brain but was later shown to be produced in peripheral tissues such as the vascular endothelium [13] and in smooth muscle cells and macrophages [14]. Because CNP plasma levels are considerably lower than those of ANP or BNP, CNP is thought to mainly act locally as a paracrine factor rather than as a circulating hormone.

Natriuretic peptide receptors

To date, three receptors for natriuretic peptides have been identified. In 1988, one type of ANP receptor was isolated from cultured vascular smooth muscle cells. Using its partial amino acid sequence, the full-length cDNA was cloned and the entire amino acid sequence was deduced [15]. The receptor molecule consists of 496 amino acid residues and contains a large extracellular domain, a putative single transmembrane helix and a 37 amino acid residue cytoplasmic domain. It is generally accepted that the role of this receptor is to bind and remove natriuretic peptides and their fragments from the circulation. Hence, this receptor is termed natriuretic peptide clearance receptor (C receptor). On the other hand, a signaling role of the C receptor has also been suggested [16].

One of the earliest events following the binding of ANP to its receptor is increase in the cytosolic cyclic guanosine monophosphate (cGMP) levels. This finding suggested that cGMP might act as the second messenger mediating the physiological activities of ANP and that the ANP receptor is coupled to guanylyl cyclase (GC), the enzyme that catalyzes the generation of cGMP. In 1989, a segment of the sea urchin GC cDNA was used as a probe to screen various cDNA libraries, which enabled cloning of the first mammalian GC (thus called GC-A) from rats and humans [17]. Expression of the cloned enzyme confirmed that GC-A is an ANP receptor. Soon after the discovery of GC-A, cloning of a second mammalian GC (GC-B) was reported [18,19]. GC-B also bound and was activated by natriuretic peptides, demonstrating the diversity within the natriuretic peptide receptor family. Since these receptor proteins were first identified as GC family members, we refer to them as GC-A or GC-B throughout this paper.

Ligand selectivity

Subsequent studies revealed that GC-A preferentially binds and responds to ANP, while GC-B preferentially responds to CNP [20]. The relative effectiveness of the three natriuretic peptides in stimulating cGMP production via GC-A and GC-B has been reported [21]. The rank order of potency for cGMP production via the GC-A receptor was ANP ≥ BNP > > CNP. On the other hand, cGMP response via GC-B was CNP > ANP or BNP. Thus, the biological functions of natriuretic peptides are mediated by two receptors: GC-A (also known as the A-type natriuretic peptide receptor, NPRA), which is selective for the cardiac peptides ANP and BNP, and GC-B (also called the B-type natriuretic peptide receptor, NPRB), which is selective for CNP.

The binding affinities of ANP, BNP and CNP to the human or rat C receptor have been reported [21]. Irrespective of the species examined, the rank order of affinity for the C receptor was ANP > CNP > BNP. This finding suggests that BNP is the least susceptible to C-receptor-mediated clearance and is more stable in the plasma.

Lessons from genetically engineered animals

A variety of genetically engineered mice have been generated to study the physiological function of each component of the natriuretic peptide–receptor system (summarized in Table 1).

Role of ANP- and BNP-mediated GC-A signaling in blood pressure regulation

Transgenic animals, which constitutively express a fusion gene consisting of the transthyretin promoter and the *ANP* gene, have plasma ANP levels that are higher than non-transgenic littermates by 5–10 fold [22]. The mean arterial pressure in the transgenic animals was reduced by 24 mmHg, which was accompanied by a 27% reduction in total heart weight. This chronic reduction in blood pressure was due to a 21% reduction in total peripheral resistance, whereas cardiac output, stroke volume and heart rate were not significantly altered. In 1994, transgenic mice carrying the human serum amyloid P component/mouse *BNP* fusion gene were generated so that the hormone expression is targeted to the liver [23]. The animals exhibited 10- to 100-fold increase in plasma BNP concentration and significantly lower blood pressure than their non-transgenic littermates.

In 1995, ANP-deficient mice were generated, and their blood pressure phenotype was reported [24]. The mutant mice (homozygous null for the *ANP* gene) had no circulating or atrial ANP, and their blood pressures were significantly higher (8–23 mmHg) than the control mice when they were fed standard diets. When fed

Table 1. Phenotypes of the genetically engineered animals for the natriuretic peptide system.

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
ANP overexpression [22]	Mouse transthyretin promoter/mouse *ANP* fusion gene	Liver	~ 25 mmHg lower than the control	27% reduction in heart weight	Plasma ANP elevated 8-fold or more; 21% reduction in peripheral resistance
ANP knockout [24]	11 bp in exon-2 replaced with the neomycin resistance gene	Systemic disruption	Increase, 8–23 mmHg (homozygotes); normal on standard diet; 27 mmHg increase on high-salt diet (heterozygotes)	Heart to body weight ratio 1.4-fold higher than the wild-type	Heterozygotes have normal level of circulating ANP
BNP overexpression [23]	Human serum amyloid P component/mouse *BNP* fusion gene	Liver	~ 20 mmHg lower than non-transgenic littermates	~ 30% less heart weight than non-transgenic littermates	10- to 100- fold increase in plasma BNP concentration; skeletal overgrowth
BNP knockout [31]	Exons 1 and 2 replaced with the neomycin resistance gene	Systemic disruption	No signs of systemic hypertension	No signs of ventricular hypertrophy; pressure-overload-induced focal ventricular fibrosis	
CNP overexpression in the cartilage [63]	Col2a1 promoter region/mouse *CNP* fusion gene	Growth plate cartilage	Not reported	Not reported	Longitudinal overgrowth of bones (limbs, vertebrae, skull)
CNP overexpression in the liver [64]	Human serum amyloid P component/mouse *CNP* fusion gene	Liver	Systolic blood pressure unaffected	Heart weight unaffected	Elongation of cartilage bones; plasma CNP level is 84% higher than control
CNP overexpression in the heart [65]	*CNP* gene fused downstream of the murine α-myosin heavy chain promoter	Heart	No change	No change at baseline	Ventricular hypertrophy after myocardial infarction is prevented
CNP knockout (Kyoto) [59]	Exons 1 and 2 encoding CNP replaced with the neomycin resistance gene	Systemic disruption	Not reported	Not reported	Severe dwarfism: impaired endochondral ossification; impaired nociceptive neurons [62]
CNP knockout (Berlin) [66]	Exon 1 replaced with a lacZ expression cassette	Systemic disruption	Not reported	Not reported	Lack of bifurcation of sensory axons in the embryonic dorsal root entry zone
GC-A knock-in overexpression [27]	Entire *GC-A* gene duplicated with the neomycin resistance gene in between	Systemic overexpression	Average 5.2 mmHg below normal in F1 mice carrying three copies of the *GC-A* gene	No effect on heart weights	
GC-A overexpression in the heart [39]	*GC-A* gene fused downstream of murine α-myosin heavy chain promoter	Heart	Normal blood pressure	Heart weight to body weight ratio was significantly less by ~ 15%	

Table 1. (Continued).

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
GC-A knockout (Dallas) [25]	Neomycin resistance gene inserted in exon 4, which encodes the transmembrane domain	Systemic disruption	Systolic blood pressure is 20–25 mmHg higher than wild-type	Global cardiac hypertrophy (40–60% increase in heart weight); cardiac contractility similar to that in wild-type mice	Rapid increases in urine output, urinary sodium and cGMP excretion after plasma volume expansion are abolished; increased susceptibility to hypoxia-induced pulmonary hypertension
GC-A knockout (North Carolina) [26]	Exon 1, intron 1 and a portion of exon 2 were replaced with the neomycin resistance gene	Systemic disruption	16 mmHg higher than the control	Heart to body weight ratio averaging185% (male) and 133% (female) of wild-type	Sudden death, with morphological evidence indicative of congestive heart failure or of aortic dissection; resistant to LPS-induced fall in blood pressure
GC-A conditional knockout	Targeting vector contains exons 1–13 and an additional 3.8 kb of the 5′ sequence of the *GC-A* gene, a loxP-flanked neomycin resistance cassette (at −2.6 kb of exon 1) and a third loxP site in the middle of intron 1	Cardiomyocytes (by crossing with cardiac α-myosin heavy chain promoter Cre mice) [43]	7–10 mmHg below normal (due to increased secretion of cardiac natriuretic peptides)	20% increase in heart to body weight ratio compared with floxed GC-A mice; ventricular collagen fractions unaffected; preserved cardiac contractility; decreased cardiac relaxation; markedly impaired cardiac function after pressure overload	~ 2-fold increase in plasma ANP concentration
		Smooth muscle cells (by crossing with SM22-Cre mice) [33]	Normal; acute effect of exogenous ANP on blood pressure abolished	Heart weight and heart to body weight ratio are not different from wild-type	Exaggerated blood pressure response to acute plasma volume expansion; higher vasodilatation sensitivity to nitric oxide and enhanced expression of soluble guanylyl cyclase
		Vascular endothelial cells (by crossing with Tie2 promoter/enhancer Cre mice) [32]	Elevated systolic blood pressure by 12–15 mmHg	~ 20% increase in heart weight	Plasma volume is increased by 11–13%; increased vascular permeability in response to ANP is abolished
GC-B dominant negative overexpression in rat [67]	Dominant-negative mutant for GC-B was fused with the CMV promoter	Whole body	No significant differences in systolic, diastolic and mean arterial pressure	Progressive cardiac hypertrophy, which was further enhanced in chronic volume overload	Reduced bone growth; modestly increased heart rate

Table 1. (Continued).

Mutated gene	Targeting construct	Targeted tissue	Blood pressure phenotype	Cardiac phenotype	Other phenotypes
GC-B dominant negative overexpression in mouse [60]	Dominant-negative mutant for GC-B, fused with promoter/enhancer regions of murine pro-α 1(II) collagen gene (Col2a1)	Cartilage	Not reported	Not reported	Significantly shorter nasoanal length
GC-B knockout [60]	Exons 3–7, encoding the C-terminal half of the extracellular ligand-binding domain and the transmembrane segment, were replaced by the neomycin resistance gene	Systemic disruption	No significant differences in blood pressure	Not reported	Impaired endochondral ossification, longitudinal vertebra or limb-bone growth; female infertility; impaired female reproductive tract development
C receptor knockout [28]	Most of exon 1 was replaced by the neomycin resistance gene	Systemic disruption	8 mmHg below normal	Not reported	Longer half-life of circulating ANP; reduced ability to concentrate urine; skeletal deformities with increased bone turnover

a standard-salt (0.5% NaCl) diet, the heterozygotes had normal circulating ANP levels and blood pressures. However, on high-salt (8% NaCl) diets, they were hypertensive, with 27 mmHg increases in systolic blood pressure levels [24].

In the same year, disruption of the *GC-A* gene was reported to result in chronically elevated blood pressure (about 25 mmHg in systolic pressure) in mice on a standard-salt diet [25]. Unlike mice heterozygous for the *ANP* gene, blood pressures of GC-A heterozygotes remained elevated and unchanged despite increasing dietary salt intake. In 1997, another group reported that the mice lacking functional *Npr1* gene, which encodes GC-A (denominated NPRA by the authors), displayed elevated blood pressure and cardiac hypertrophy with interstitial fibrosis resembling that seen in human hypertensive heart disease [26]. In a subsequent paper, the blood pressures of one-copy F1 animals were reported to be significantly higher on high-salt diet than on low-salt diet [27]. The reason for the discrepancy between the salt phenotypes of these two *GC-A* knockout mouse strains is still unknown. It is possible that differences result from different targeting strategies or the genetic background of the mouse strains used.

In 1999, the generation of mice in which the C receptor was inactivated by homologous recombination was reported [28]. C-receptor-deficient mice have less ability to concentrate urine, exhibit mild diuresis and tend to have depleted blood volume. C receptor homozygous mutants have significantly lower blood pressures (by 8 mmHg) than their wild-type counterparts. The half-life of ANP in C-receptor-deficient mice is two-thirds longer than that in wild-type mice, demonstrating that C receptor plays a significant role in its clearance. Moreover, C receptor modulates the availability of the natriuretic peptides to their target organs, thereby allowing the activity of the natriuretic peptide system to be tailored to specific local needs. In fact, C receptor expression is tightly regulated by other signaling molecules, such as angiotensin II [29] and catecholamines [30]. Interestingly, the baseline levels of ANP and BNP were not higher in the C-receptor-deficient mice than in the wild-type mice, implying that either the cardiac secretion or C-receptor-independent clearance mechanism was altered in those mice.

In 2000, the targeted disruption of the *BNP* gene in mice was reported. Multifocal fibrotic lesions were found in the ventricles of BNP-deficient mice, suggesting the protective role of BNP in pathological cardiac fibrosis [31]. Interestingly, there were no signs of systemic hypertension or ventricular hypertrophy, suggesting that in the presence of ANP basal levels of BNP are dispensable for these cardiovascular phenotypes.

To examine the tissue(s) responsible for the hypertensive phenotype of systemic GC-A-null mice, a targeting strategy was designed so that Cre recombinase mediates the deletion of exon 1 of the *GC-A* gene. Thus, in floxed GC-A mice, GC-A can be deleted in a tissue-specific manner. Endothelium-specific deletion of GC-A was achieved by crossing the floxed GC-A mice with transgenic mice expressing Cre recombinase under the control of the Tie2 promoter/enhancer. Endothelium-specific GC-A-deficient mice display significantly increased systolic blood pressure (by approximately 12–15 mmHg) and diastolic blood pressure (by approximately 5–10 mmHg) than their control littermates [32]. Interestingly, although the direct vasodilatation effects of exogenously administered ANP were abolished, smooth-muscle-cell-restricted deletion of GC-A did not affect the resting blood pressure [33], indicating that endothelial cell GC-A, and not vascular smooth muscle cell GC-A, is indispensable for chronic regulation of blood pressure.

Overall, these results show the significance of the endogenous natriuretic peptide system in the maintenance of normal blood pressure.

Regulation of blood volume

Infusion of ANP results in substantial natriuresis and diuresis in wild-type mice but fails to cause significant changes in sodium excretion or urine output in GC-A-deficient mice, indicating that GC-A is essential for ANP-induced acute regulation of diuresis and natriuresis [34]. After experimental expansion of the plasma volume, urine output as well as urinary sodium and cGMP excretion increase rapidly and markedly in the wild-type but not in systemic GC-A-deficient animals. Nevertheless, plasma ANP levels are comparable or even higher in CG-C-deficient animals [34]. On the contrary, the knock-in overexpression of GC-A (four-copy) in mice results in augmented responses to volume expansion in urinary flow and sodium excretion along with rises in both glomerular filtration rate and renal plasma flow, compared with wild-type (two-copy) mice after volume expansion [35]. These results establish that GC-A activation is the predominant mechanism mediating the natriuretic, diuretic and renal hemodynamic responses to acute blood volume expansion.

The plasma volumes of animals completely lacking GC-A are expanded by 30%, suggesting the role of GC-A in chronic regulation of the blood volume. Interestingly, mice lacking GC-A specifically in the vascular endothelium are volume expanded by 11–13% [32], suggesting that GC-A in the endothelium at least partly accounts for chronic blood volume regulatory effects. Since previous experiments indicated that ANP increased capillary permeability of the endothelium to macromolecules like albumin [36], these data suggest that the ANP/GC-A pathway regulates chronic transvascular fluid balance by increasing microvascular permeability [37].

Cardiac remodeling and the local natriuretic peptide system

Cardiac synthesis and secretion of ANP and BNP are increased according to the severity of cardiac remodeling in humans as well as in animal models [38]. Since the two cardiac natriuretic peptides share a common receptor (i.e. GC-A), the cardiac phenotype of mice lacking GC-A revealed complete effects of the cardiac natriuretic peptide signaling. Notably, targeted deletion of the *GC-A* gene resulted in marked cardiac hypertrophy and fibrosis, which were disproportionately severe [39,40] given the modest rise in blood pressure [25]. Since the chronic treatment of GC-A-deficient mice with anti-hypertensive drugs, which reduce blood pressure to levels similar to those seen in wild-type mice, has no significant effect on cardiac hypertrophy [41], these results imply that the natriuretic peptides/GC-A system has direct anti-hypertrophic effects in the heart, which are independent of its roles in blood pressure and body fluid control.

More direct evidence of local anti-hypertrophic GC-A signaling was obtained from animals in which the *GC-A* gene was conditionally targeted. The *GC-A* gene was selectively overexpressed in the cardiomyocytes of wild-type or *GC-A*-null animals, and the effects were examined [39]. Whereas introduction of the *GC-A* transgene did not alter blood pressure or heart rate as a function of genotype, it did reduce cardiomyocyte size in both wild-type and null backgrounds. The reduction in myocyte size was accompanied by a decrease in cardiac ANP mRNA expression, which suggests the existence of a local regulatory mechanism that governs cardiomyocyte size and gene expression via a GC-A-mediated pathway [42]. Conversely, the *GC-A* gene was inactivated selectively in cardiomyocytes by homologous loxP/Cre-mediated recombination, which circumvents the systemic hypertensive phenotype associated with germline disruption of the *GC-A* gene [43]. Mice with cardiomyocyte-restricted GC-A deletion exhibited mild cardiac hypertrophy with markedly increased transcription of cardiac hypertrophy markers, including ANP. These observations are consistent with the idea that a local function of the ANP/GC-A system is to moderate the molecular program of cardiac hypertrophy [44].

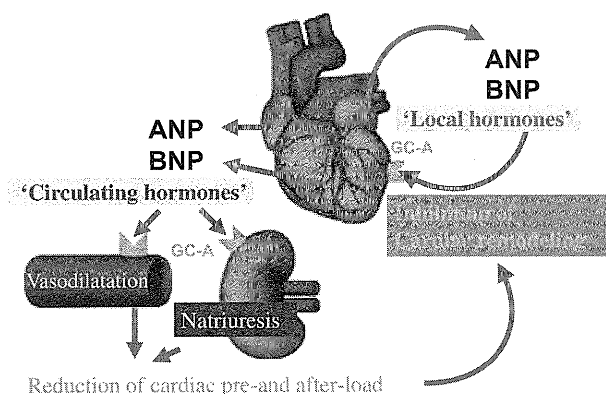

Fig. 1. ANP and BNP, the cardiac natriuretic peptides, protect the heart in not only an endocrine but also a paracrine fashion. Because ANP and BNP have potent diuretic, natriuretic and vasodilatory actions, augmentation of the ANP and BNP/GC-A signaling leads to a decrease in cardiac pre- and after-load, and their mobilization during cardiac failure is considered one of the compensatory mechanisms activated in response to heart damage. In addition to the hemodynamic effects of their actions as circulating hormones, recent evidence suggests that ANP and BNP also exert local cardioprotective effects by acting as autocrine/paracrine hormones.

Since the diuretic, natriuretic and vasorelaxant activities of ANP and BNP lead to reduction of the cardiac pre- and after-load, these results suggest that the cardiac natriuretic peptides/GC-A signaling exerts its cardioprotective actions in both an endocrine and an autocrine/paracrine fashion. These mechanisms are schematically depicted in Fig. 1.

The molecular mechanism of GC-A-mediated inhibition of cardiac hypertrophy

To identify the molecular mechanism underlying cardiac hypertrophy seen in GC-A-deficient mice, DNA microarrays were used to identify genes upregulated in the hypertrophied heart [45]. Among several genes known to be upregulated in cardiac hypertrophy (e.g. α-skeletal actin, ANP and BNP), it has been found that the expression of the gene encoding myocyte-enriched calcineurin-interacting protein (MCIP1) is also increased. The *MCIP1* gene is reportedly regulated by calcineurin, a critical regulator of cardiac hypertrophy. Thus, it was hypothesized that the calcineurin activity is enhanced in the heart of GC-A-deficient mice. To test this hypothesis, cultured neonatal cardiomyocytes were used to determine whether pharmacological inhibition of GC-A would increase calcineurin activity, which it did not [45]. On the other hand, stimulation of GC-A with ANP inhibited calcineurin activity, suggesting that it is by inhibiting the calcineurin pathway that cardiac GC-A signaling (activated by locally secreted natriuretic peptides) exerts its anti-hypertrophic effects. In fact, chronic treatment with FK506, which in combination with FK506-binding protein inhibits the phosphatase activity of calcineurin, significantly reduces the heart weight to body weight ratio, cardiomyocyte size and collagen volume fraction in GC-A-deficient mice compared with the wild-type mice [45]. A further study using microarray analysis and real-time PCR analysis revealed that, in addition to the calcineurin–nuclear factor of activated T-cells (NFAT) pathway, the calmodulin–CaMK–Hdac–Mef2 and PKC–MAPK–GATA4 pathways may also be involved in the cardiac hypertrophy seen in the GC-A-null mice [46].

Role of regulator of G-protein signaling in CG-A cardioprotective actions

Recently, it has been elegantly demonstrated that cGMP-dependent protein kinase (PKG) Iα attenuates signaling by the thrombin receptor protease-activated receptor (PAR) 1 through direct activation of regulator of G-protein signaling (RGS) 2 [47]. PKG-Iα binds directly to and phosphorylates RGS-2, which significantly increases the GTPase activity of $G\alpha_q$, thereby terminating PAR-1 signaling. Given that cGMP is an intracellular second messenger for natriuretic peptides, RGS might mediate the cardioprotective effect of the GC-A signaling. To test this hypothesis, the role of RGS-4, which is the predominant RGS in cardiomyocytes under physiological conditions, was examined. In cultured cardiomyocytes, ANP stimulated the binding of PKG-Iα to RGS-4 as well as the phosphorylation of RGS-4 and its subsequent association with $G\alpha_q$ [48]. In addition, cardiomyocyte-specific overexpression of RGS-4 in GC-A-null mice significantly rescued the cardiac phenotype of these mice. On the contrary, overexpression of a dominant-negative form of RGS-4 blocked the inhibitory effects of ANP on cardiac hypertrophy [48]. Therefore, GC-A may activate cardiac RGS-4, which then inhibits the activity of $G\alpha_q$ and its downstream hypertrophic effectors. The endogenous cardioprotective mechanism meditated by ANP/BNP, GC-A and RGS-4 is depicted schematically in Fig. 2.

Very recently, PKG activation reflecting chronic inhibition of cGMP-selective phosphodiesterase 5 has been shown to suppress maladaptive cardiac hypertrophy by inhibiting $G\alpha_q$-coupled stimulation, and the effect was not observed in mice lacking RGS-2 [49]. This suggests that RGS2 mediates the cardioprotective actions of PKG in pathological conditions such as

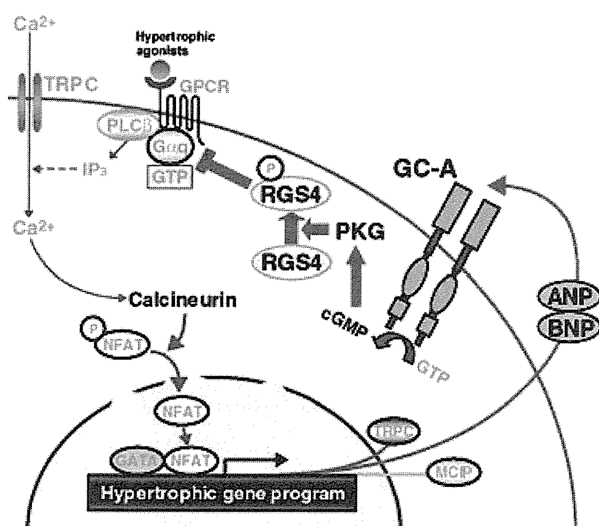

Fig. 2. Inhibitory mechanism of cardiac hypertrophy by the local natriuretic peptide system. Cardiac hypertrophy agonists such as angiotensin II, catecholamines and endothelins stimulate G-protein coupled receptor. Subsequent production of inositol triphosphate (IP3) promotes elevation of intracellular Ca^{2+} levels, which results in activation of the calcineurin/nuclear factor of activated T cells (NFAT) pathway. Cooperatively with the family of GATA transcription factors, NFAT activates the hypertrophic gene program, which includes the ANP- and BNP-coding genes. In an autocrine or paracrine fashion, ANP and BNP stimulate their receptor GC-A and exert their anti-hypertrophic actions via the activation of the RGS, which consequently results in an increase in the GTPase activity of the α subunit of the guanine nucleotide binding protein ($G\alpha_q$) and in a decrease in the activity of the downstream signaling mediators (adapted from [48]).

pressure overload or excessive $G\alpha_q$ activation due to hypertrophic stimuli. In fact, RGS-2 is also implicated in the anti-hypertrophic action of cardiac GC-A [50].

The role of GC-A in myocardial infarction

It is well known that plasma levels of ANP and BNP are dramatically elevated early after myocardial infarction [51]. To examine the significance of this upregulation, experimental myocardial infarction by ligation of the left coronary artery was induced in mice lacking GC-A [52]. GC-A-deficient mice exhibited significantly higher mortality rate than wild-type mice, reflecting a higher incidence of acute heart failure. Four weeks after infarction, left ventricular remodeling, including myocardial hypertrophy and fibrosis, and impairment of the left ventricular systolic function were significantly more severe in mice lacking GC-A than in wild-type mice [52]. GC-A activation by endogenous cardiac natriuretic peptides may protect against acute heart failure and attenuate chronic cardiac remodeling after acute myocardial infarction.

Role of GC-A in peripheral arterial disease

A role of the natriuretic peptide system in peripheral arterial diseases has also been suggested. Activation of the natriuretic peptides–cGMP–PKG pathway was found to accelerate vascular regeneration and blood flow recovery in a murine model of peripheral arterial disease, in which leg ischemia was induced by femoral arterial ligation [53]. Recently, it has been reported that intraperitoneal injection of carperitide, a recombinant human ANP, accelerated blood flow recovery with increasing capillary density in the ischemic legs [54], indicating the role of exogenously administered ANP and BNP in angiogenesis. When the hindlimb ischemia model was performed in GC-A-deficient mice, autoamputation or ulcers were more severe in GC-A-deficient mice than in their wild-type counterparts [55]. Laser Doppler perfusion imaging revealed that the recovery of blood flow in the ischemic limb was significantly inhibited in GC-A-null mice compared with wild-type mice. In addition, vascular regeneration in response to critical hindlimb ischemia was severely impaired [55]. Similar attenuation of ischemic angiogenesis was observed in mice with conditional, endothelial-cell-restricted GC-A deletion. On the other hand, smooth-muscle-cell-restricted GC-A ablation did not affect ischemic neovascularization [56], suggesting that it is the endothelial GC-A that stimulates endothelial regeneration after induction of ischemia. Taken together, the evidence suggests that the natriuretic peptide pathway significantly contributes to peripheral vascular remodeling during ischemia.

Role of the CNP/GC-B pathway in bone formation

In a 1998 study, mice with transgenic overexpression of the *BNP* gene, especially those exhibiting high expression levels, unexpectedly displayed deformed bony skeletons characterized by kyphosis, elongated limbs and paws, and crooked tails, which resulted from a high turnover of endochondral ossification accompanied by overgrowth of the growth plate [57]. Even after crossing with GC-A-null mice, transgenic mice overexpressing BNP continued to exhibit marked longitudinal growth of the vertebrae and long bones [58]. Therefore, the effect of excess amount of BNP on endochondral ossification is independent of GC-A, and so signaling through another receptor was suggested.