Table 5
Anthropometric, hemodynamic, and cardiometabolic parameters in elderly hypertensives with or without abdominal obesity.

	Total subjects (n = 263)		P value
	Non-obesity (n = 136)	Abdominal obesity (n = 127)	
Age (years)	73.3 ± 8.2	71.9 ± 8.7	0.153
Men, n (%)	35 (26)	61 (48)	<0.001
BMI (kg/m^2)	22.3 ± 2.3	26.6 ± 3.1	<0.001
Waist (cm)	79.3 ± 6.1	93.1 ± 7.0	<0.001
A BP measurement			
24-h SBP (mmHg)	131.8 ± 12.7	138.3 ± 17.2	0.001
24-h DBP (mmHg)	76.5 ± 7.1	78.9 ± 8.3	0.010
Laboratory testing			
Insulin (μIU/ml)	4.8 (3.2–6.6)	6.9 (5.1–10.6)	<0.001
Triglycerides (mg/dl)	88.0 (68.0–119.8)	107.0 (79.0–154.0)	<0.001
hs-CRP (mg/l)	0.44 (0.21–0.93)	0.66 (0.36–1.28)	0.004
PAI-1 (ng/ml)	32.0 (27.0–40.0)	38.0 (30.0–54.0)	0.001
NO_x (μmol/l)	33.0 (23.0–50.0)	31.0 (23.0–51.0)	0.974
HMW adiponectin (μg/ml)	7.8 (5.0–11.3)	5.2 (3.5–8.9)	<0.001
Des-acyl ghrelin (fmol/ml)	116.8 (81.4–193.9)	98.3 (70.7–140.5)	0.003
cIMT (mm)	0.782 ± 0.163	0.833 ± 0.185	0.019

Data are expressed as means ± S.D. or median (25th to 75th percentile). Statistical significance was defined as $P < 0.05$.

dicting atherosclerosis in elderly hypertensives. Although there was a significant association between the des-acyl ghrelin and NO_x levels, the association of des-acyl ghrelin with atherosclerosis appears to be independent of the NO_x level. Because of previous experimental studies in which the cardiovascular protective effect of des-acyl ghrelin was suggested to be robust [12–14], the hypothesis that des-acyl ghrelin protects against the development of atherosclerosis is attractive. Our data warrant further investigation of the pathologic mechanisms responsible for this phenomenon and to clarify the prognostic value of this peptide with respect to cardiovascular events in the future.

4.1. Effects of HMW adiponectin on atherosclerosis in elderly hypertensives

Although the cardiovascular risks of obesity in elderly persons are still debatable [1,7], our data showed that elderly persons with abdominal obesity had a significantly higher level of cIMT. The mechanisms of this relation were not explained by the HMW adiponectin level or its related cardiometabolic parameters (glucose and lipid metabolites, inflammation, and hemostasis), despite the fact that the absolute magnitude of their difference between those with and without abdominal obesity was larger than for the des-acyl ghrelin level. Furthermore, the HMW adiponectin level could not predict the higher level of cIMT. These observations raised at least two possibilities. First, the detrimental effects of these parameters on atherosclerosis are generally weaker in elderly than middle-aged persons [1,5,8], which may be partially explained by the survival-effects [18]. Second, because HMW adiponectin could be influenced by many physiological and pathophysiological factors, interpretation of the HMW adiponectin level in elderly persons should be undertaken with caution. In fact, our study showed that the significant inverse association between HMW adiponectin and obesity [3–5] becomes attenuated with aging (≥75 years). This may be partly explained by the fact that the HMW adiponectin level is proportionally increased with age and impaired renal function, and thus an individual difference in advanced aging might become inconspicuous. Furthermore, our data show a significant inverse correlation between weight change from 20 years of age to current weight and HMW adiponectin levels (data not shown), suggesting a possibility that HMW adiponectin levels among the elderly are modulated by systemic energy valance [5–7]. Because weight decline (called *wasting*) is associated with an adverse cardiovascular outcome in elderly persons [8], high levels of HMW adiponectin might also reflect harmful signals in the body in advanced age [5,6].

4.2. Des-acyl ghrelin and atherosclerosis in elderly hypertensives

In contrast to HMW adiponectin, the inverse correlation between des-acyl ghrelin and obesity was particularly strong in our subjects with advanced aged (≥75 years). The mechanisms responsible for the reduction of des-acyl ghrelin in obesity remain unknown, but at least two possible explanations could be considered. First, the decreased activity of acylation enzyme or increased activity of endogenous esterase may occur in obesity. Barazzoni et al. reported that abdominal fat accumulation leads to accelerated ghrelin acylation in conjunction with a decrease in the des-acyl ghrelin levels [19]. Our study did not measure the plasma level of acylated ghrelin, because the measurement of this parameter is technically complex (e.g., acidified plasma with a 1/10 volume of 1N HCl is needed), and thus this hypothesis remains untested. Second, the decreased des-acyl ghrelin level may be a consequence of unmeasured abnormal adipochemokines or physiological adaptation to the positive energy balance associated with obesity. Further research is needed in this area.

In the current study, the des-acyl ghrelin level showed a negative correlation with cIMT independently of age, WC, sex, smoking, 24-h BP and renal function. Because the des-acyl ghrelin level parallels the acylated ghrelin level [15], our data may simply confirm those of the previous report in which decreased total ghrelin levels were associated with the progression of cIMT in elderly patients with metabolic syndrome [20]. However, numerous studies suggest that des-acyl ghrelin itself shows a wide array of cardiovascular activities [10–14], and thus des-acyl ghrelin may exert independent effects on the cardiovascular system. The pathologic pathways linking des-acyl ghrelin and cIMT remain unclear, and are probably not related to factors such as hemodynamic, metabolic or inflammatory pathways. That des-acyl ghrelin was significantly correlated only with the NO_x level suggests the possibility that des-acyl ghrelin may increase NO bioactivity. This consideration is supported by the recent report of Tesauro et al., which demonstrated that administration of des-acyl ghrelin reversed endothelial dysfunction by increasing NO bioactivity [13]. Des-acyl ghrelin is as effective as ghrelin in activating intracellular signaling pathways (i.e., ERK-

1/2, Akt) [14] that could be involved in endothelial NO production. However, our data indicate that the association of des-acyl ghrelin with atherosclerosis is independent from the NO_x level, thus the mechanisms responsible for this phenomenon could not be clarified in the present investigation. Nevertheless, because previous experiments showed a cardiovascular protective effect of des-acyl ghrelin [12–14], it is feasible that des-acyl ghrelin protects against the development of atherosclerosis. The atherosclerotic risks of abdominal obesity in our elderly persons were largely explained by elevated 24-h BP and reduced des-acyl ghrelin levels, suggesting the possibility that an increase in the des-acyl ghrelin level, in addition to BP control, may counteract the obesity-related atherosclerosis in elderly persons.

Our study has several limitations. First, because of the cross-sectional nature of our data, we cannot infer any causality. Hopefully longitudinal follow-up data will be able to provide some insights. Second, because our study was conducted in elderly hypertensives, caution should be used in applying the results to different groups. Third, all cardiometabolic parameters were measured only once. Finally, medication use may be potentially confounding, although our results were not changed after adjustment of these factors as a covariate.

In conclusion, the current study has demonstrated that the plasma des-acyl ghrelin level is a suitable predictor of cardiovascular risk in elderly hypertensives. The prognostic value of this peptide with respect to cardiovascular events will be addressed in a follow-up study in the present study population. In addition, the cardiovascular protective effect of des-acyl ghrelin suggests that the peptide could play a modulating role in atherosclerosis, especially in obese subjects. Further intervention studies will be needed to clarify this possibility.

Acknowledgments

This study was partly supported by a grant-in-aid from the Foundation for the Development of the Community (Y.Y.). The authors are grateful for the dedicated work of our staff. We also thank T. Nagatomo, K. Kai, E. Kusao, M. Kawano, T. Hidaka, G. Nakamura, and S. Matsuda for their excellent support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.013.

References

[1] Heiat A, Vaccarino V, Krumholz HM. An evidence-based assessment of federal guidelines for overweight and obesity as they apply to elderly persons. Arch Intern Med 2001;161:1194–203.
[2] Trujillo ME, Scherer PE. Adipose tissue-derived factors: impact on health and disease. Endocrinol Rev 2006;27:762–78.
[3] Matsuzawa Y, Funahashi T, Kihara S, Shimomura I. Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol 2004;24:29–33.
[4] Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. JAMA 2004;291:1730–7.
[5] Wannamethee SG, Whincup PH, Lennon L, Sattar N. Circulating adiponectin levels and mortality in elderly men with and without cardiovascular disease and heart failure. Arch Intern Med 2007;167:1510–7.
[6] Kadowaki T, Yamauchi T, Kubota N. The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. FEBS Lett 2008;582:74–80.
[7] Kubota N, Yano W, Kubota T, et al. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 2007;6:55–68.
[8] Wedick NM, Barrett-Connor E, Knoke JD, Wingard DL. The relationship between weight loss and all-cause mortality in older men and women with and without diabetes mellitus: the Rancho Bernardo study. J Am Geriatr Soc 2002;50:1810–5.
[9] Pajvani UB, Du X, Combs TP, et al. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem 2003;278:9073–85.
[10] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–60.
[11] Nakazato M, Murakami N, Date Y, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–8.
[12] Kleinz MJ, Maguire JJ, Skepper JN, Davenport AP. Functional and immunocytochemical evidence for a role of ghrelin and des-octanoyl ghrelin in the regulation of vascular tone in man. Cardiovasc Res 2006;69:227–35.
[13] Tesauro M, Schinzari F, Iantorno M, et al. Ghrelin improves endothelial function in patients with metabolic syndrome. Circulation 2005;112:2986–92.
[14] Baldanzi G, Filigheddu N, Cutrupi S, et al. Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 2003;159:1029–37.
[15] Akamizu T, Shinomiya T, Irako T, et al. Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. J Clin Endocrinol Metab 2005;90:6–9.
[16] Otsuki M, Hashimoto K, Morimoto Y, Kishimoto T, Kasayama S. Circulating vascular cell adhesion molecule-1 (VCAM-1) in atherosclerotic NIDDM patients. Diabetes 1997;46:2096–101.
[17] The committee to evaluate diagnostic standards for metabolic syndrome 2005 Definition and the diagnostic standard for metabolic syndrome. Nippon Naika Gakkai Zasshi 2005;94:794–809 [in Japanese].
[18] Inelmen EM, Sergi G, Coin A, Miotto F, Peruzza S, Enzi G. Can obesity be a risk factor in elderly people? Obes Rev 2003;4:147–55.
[19] Barazzoni R, Zanetti M, Ferreira C, et al. Relationships between desacylated and acylated ghrelin and insulin sensitivity in the metabolic syndrome. J Clin Endocrinol Metab 2007;92:3935–40.
[20] Kotani K, Sakane N, Saiga K, et al. Serum ghrelin and carotid atherosclerosis in older Japanese people with metabolic syndrome. Arch Med Res 2006;37:903–6.

Biochemical and Biophysical Research Communications 389 (2009) 405–408

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ghrelin reverses experimental diabetic neuropathy in mice

Itaru Kyoraku [a], Kazutaka Shiomi [a], Kenji Kangawa [b], Masamitsu Nakazato [a,*]

[a] Division of Neurology, Respirology, Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan
[b] Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

ARTICLE INFO

Article history:
Received 13 August 2009
Available online 3 September 2009

Keywords:
Ghrelin
Diabetic neuropathy
Nerve conduction velocity
Thermal sensation
Oxidative stress

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.

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

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.171

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.

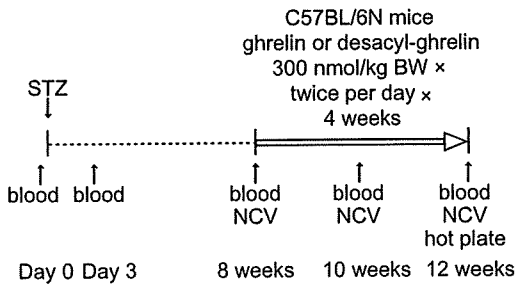

Fig. 1. Schematic representation of the experimental schedule.

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.

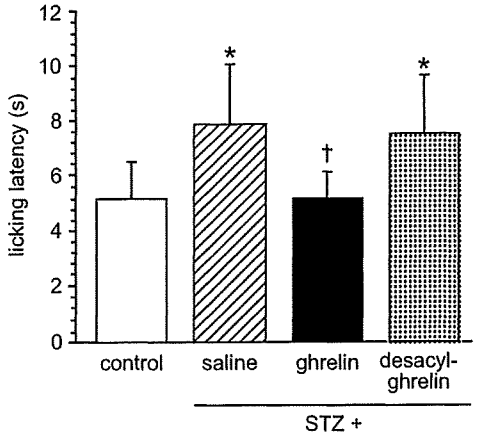

Fig. 3. Effect of ghrelin on thermal sensation. Ghrelin administration significantly improved licking latency in the hot plate test compared with saline administration. *$P < 0.01$ versus controls. †$P < 0.01$ versus STZ + saline and STZ + desacyl-ghrelin. A one-way ANOVA with Bonferroni post hoc *t*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

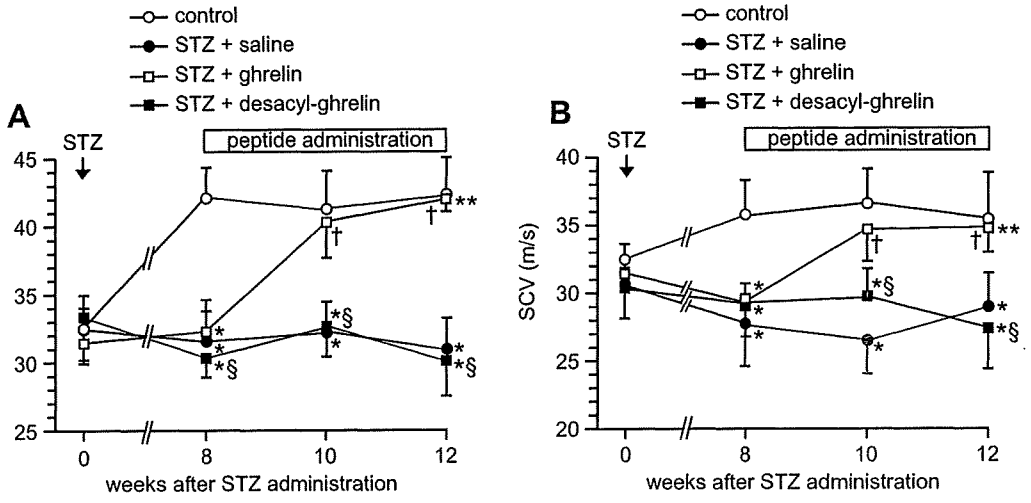

Fig. 2. Effect of ghrelin on NCV. MCV (A) and SCV (B) were reduced significantly after STZ treatment. Ghrelin administration, but not desacyl-ghrelin, ameliorated the decreases in both MCV and SCV 2 weeks after the start of administration. Data are expressed as means ± SE. *$P < 0.001$ versus controls. #$P < 0.001$ 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.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (B) No. 19390254, Grants-in-Aid for Exploratory Research No. 20013038, the Japan Society for the Promotion of Science (JSPS), the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Grant-in-Aid for Comprehensive Research on Aging and Health No. H19-Choujyu-Ippan-022, the Ministry of Health Labour and Welfare, and Takeda Science Foundation.

References

[1] P.J. Dyck, K.M. Kratz, J.L. Karnes, W.J. Litchy, R. Klein, J.M. Pach, D.M. Wilson, P.C. O'Brien, L.J. Melton 3rd., F.J. Service, The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study, Neurology 43 (1993) 817–824.
[2] A.I. Vinik, T.S. Park, K.B. Stansberry, G.L. Pittenger, Diabetic neuropathies, Diabetologia 43 (2000) 957–973.
[3] F. Roy, The nervous system and diabetes, in: C.R. Kahn, G.C. Weir, G.L. King, A.M. Jacobsen, A.C. Moses, R.J. Smith (Eds.), Joslin's Diabetes Mellitus, 14th ed., Lippincott Williams & Wilkins, Philadelphia, 2005, pp. 951–968.
[4] P.A. Low, Recent advances in the pathogenesis of diabetic neuropathy, Muscle Nerve 10 (1987) 121–128.
[5] N.E. Cameron, M.A. Cotter, Metabolic and vascular factors in the pathogenesis of diabetic neuropathy, Diabetes 46 (Suppl. 2) (1997) S31–S37.
[6] P. Rösen, P.P. Nawroth, G. King, W. Möller, H.J. Tritschler, L. Packer, The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society, Diabetes Metab. Res. Rev. 17 (2001) 189–212.
[7] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature 402 (1999) 656–660.
[8] Y. Date, K. Toshinai, S. Koda, M. Miyazato, T. Shimbara, T. Tsuruta, A. Niijima, K. Kangawa, M. Nakazato, Peripheral interaction of ghrelin with cholecystokinin on feeding regulation, Endocrinology 146 (2005) 3518–3525.
[9] M. Tschöp, D.L. Smiley, M.L. Heiman, Ghrelin induces adiposity in rodents, Nature 407 (2000) 908–913.
[10] M. Nakazato, N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, S. Matsukura, A role for ghrelin in the central regulation of feeding, Nature 409 (2001) 194–198.
[11] M. Kojima, K. Kangawa, Drug Insight: the functions of ghrelin and its potential as a multitherapeutic hormone, Nat. Clin. Pract. Endocrinol. Metab. 2 (2006) 80–88.
[12] P. Schratzberger, D.H. Walter, K. Rittig, F.H. Bahlmann, R. Pola, C. Curry, M. Silver, J.G. Krainin, D.H. Weinberg, A.H. Ropper, J.M. Isner, Reversal of experimental diabetic neuropathy by VEGF gene transfer, J. Clin. Invest. 107 (2001) 1083–1092.
[13] Y.S. Chen, S.S. Chung, S.K. Chung, Noninvasive monitoring of diabetes-induced cutaneous nerve fiber loss and hypoalgesia in *thy1*-YFP transgenic mice, Diabetes 54 (2005) 3112–3118.
[14] B. Kakinoki, S. Sekimoto, S. Yuki, T. Ohgami, M. Sejima, K. Yamagami, K. Saito, Orally active neurotrophin-enhancing agent protects against dysfunctions of the peripheral nerves in hyperglycemic animals, Diabetes 55 (2006) 616–621.
[15] N.M. Neary, C.J. Small, A.M. Wren, J.L. Lee, M.R. Druce, C. Palmieri, G.S. Frost, M.A. Ghatei, R.C. Coombes, S.R. Bloom, Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial, J. Clin. Endocrinol. Metab. 89 (2004) 2832–2836.
[16] N. Nagaya, T. Itoh, S. Murakami, H. Oya, M. Uematsu, K. Miyatake, K. Kangawa, Treatment of cachexia with ghrelin in patients with COPD, Chest 128 (2005) 1187–1193.
[17] D. Miljic, S. Pekic, M. Djurovic, M. Doknic, N. Milic, F.F. Casanueva, M. Ghatei, V. Popovic, Ghrelin has partial or no effect on appetite, growth hormone, prolactin, and cortisol release in patients with anorexia nervosa, J. Clin. Endocrinol. Metab. 91 (2006) 1491–1495.
[18] T. Kodama, J. Ashitani, N. Matsumoto, K. Kangawa, M. Nakazato, Ghrelin treatment suppresses neutrophil-dominant inflammation in airways of patients with chronic respiratory infection, Pulm. Pharmacol. Ther. 21 (2008) 774–779.
[19] F. Strasser, T.A. Lutz, M.T. Maeder, B. Thuerlimann, D. Bueche, M. Tschöp, K. Kaufmann, B. Holst, M. Brändle, R. von Moos, R. Demmer, T. Cerny, Safety, tolerability and pharmacokinetics of intravenous ghrelin for cancer-related anorexia/cachexia: a randomised, placebo-controlled, double-blind, double-crossover study, Br. J. Cancer 98 (2008) 300–308.
[20] P.A. Low, K.K. Nickander, H.J. Tritschler, The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy, Diabetes 46 (Suppl. 2) (1997) S38–S42.
[21] H. Zhao, G. Liu, Q. Wang, L. Ding, H. Cai, H. Jiang, Z. Xin, Effect of ghrelin on human endothelial cells apoptosis induced by high glucose, Biochem. Biophys. Res. Commun. 326 (2007) 677–681.
[22] E.E. Eter, A.A. Tuwaijiri, H. Hagar, M. Arafa, *In vivo* and *in vitro* antioxidant activity of ghrelin: attenuation of gastric ischemic injury in the rat, J. Gastroenterol. Hepatol. 22 (2007) 1791–799.
[23] G. Baldanzi, N. Filigheddu, S. Cutrupi, F. Catapano, S. Bonissoni, A. Fubini, D. Malan, G. Baj, R. Granata, F. Broglio, M. Papotti, N. Surico, F. Bussolino, J. Isgaard, R. Deghenghi, F. Sinigaglia, M. Prat, G. Muccioli, E. Ghigo, A. Graziani, Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT, J. Cell Biol. 159 (2004) 1029–1037.
[24] N.M. Thompson, D.A. Gill, R. Davies, N. Loveridge, P.A. Houston, I.C. Robinson, T. Wells, Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor, Endocrinology 145 (2004) 234–242.
[25] G. Muccioli, N. Pons, C. Ghè, F. Catapano, R. Granata, E. Ghigo, Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor, Eur. J. Pharmacol. 498 (2004) 27–35.
[26] A.M. Nanzer, S. Khalaf, A.M. Mozid, R.C. Fowkes, M.V. Patel, J.M. Burrin, A.B. Grossman, M. Korbonits, Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway, Eur. J. Endocrinol. 151 (2004) 233–240.
[27] P.J. Delhanty, B.C. van der Eerden, M. van der Velde, C. Gauna, H.A. Pols, H. Jahr, H. Chiba, A.J. van der Lely, J.P. van Leeuwen, Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a, J. Endocrinol. 188 (2006) 37–47.
[28] K. Toshinai, H. Yamaguchi, Y. Sun, R.G. Smith, A. Yamanaka, T. Sakurai, Y. Date, M.S. Mondal, T. Shimbara, T. Kawagoe, N. Murakami, M. Miyazato, K. Kangawa, M. Nakazato, Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor, Endocrinology 147 (2006) 2306–2314.
[29] N. Filigheddu, V.F. Gnocchi, M. Coscia, M. Cappelli, P.E. Porporato, R. Taulli, S. Traini, G. Baldanzi, F. Chianale, S. Cutrupi, E. Arnoletti, C. Ghè, A. Fubini, N. Surico, F. Sinigaglia, C. Ponzetto, G. Muccioli, T. Crepaldi, A. Graziani, Ghrelin and des-acyl ghrelin promote differentiation and fusion of C2C12 skeletal muscle cells, Mol. Biol. Cell 18 (2007) 986–994.

Nutrition Journal

BioMed Central

Short report

Open Access

Effect of octanoic acid-rich formula on plasma ghrelin levels in cachectic patients with chronic respiratory disease

Jun-ichi Ashitani*, Nobuhiro Matsumoto and Masamitsu Nakazato

Address: Third Department of Internal Medicine, Miyazaki University School of Medicine, Kihara 5200, Miyazaki 889-1692, Japan

Email: Jun-ichi Ashitani* - jashi2@fc.miyazaki-u.ac.jp; Nobuhiro Matsumoto - nobu@fc.miyazaki-u.ac.jp; Masamitsu Nakazato - nakazato@fc.miyazaki-u.ac.jp

* Corresponding author

Published: 16 June 2009

Nutrition Journal 2009, 8:25 doi:10.1186/1475-2891-8-25

Received: 12 March 2009
Accepted: 16 June 2009

This article is available from: http://www.nutritionj.com/content/8/1/25

Abstract

Background: For cachectic patients with chronic respiratory disease (CRD), conventional enteral nutrition formula is an optional treatment to maintain energy balance. The molecular mechanisms by which enteral nutrition formula controls appetite and weight remain unknown. We examined whether enteral nutrition formula rich in octanoic acids would increase plasma levels of ghrelin, an appetite-stimulating hormone produced in the stomach, in cachectic patients with CRD.

Methods: Plasma ghrelin profiles in cachectic patients with CRD were assessed and compared with those in age- and sex-matched controls. Plasma levels of acyl-ghrelin, an active ghrelin modified by octanoic acids, and desacyl-ghrelin were measured separately. We examined changes in 24-h plasma ghrelin profiles before and after single administration of the formula. We also evaluated the effects of 2-week administration of the formula on plasma ghrelin levels and nutritional status in patients.

Results: The ratio of acyl-ghrelin to desacyl-ghrelin in plasma was lower in patients than in controls. Single administration of the formula did not change plasma desacyl-ghrelin levels, but induced an increase in acyl-ghrelin levels. Two-week treatment with the formula was effective in increasing weight and acyl-ghrelin, along with improving nutritional status in patients.

Conclusion: These results show that the formula contributes to increased weight, which may be associated with induction of acyl-ghrelin production in cachectic patients with CRD.

Background

Weight loss and nutritional depletion represent independent risk factors for the incidence of pneumonia and mortality in patients with chronic respiratory diseases (CRD) [1,2]. Excess energy expenditure and appetite loss are the main causes of weight loss in such patients, and are difficult to control using established treatments. Enteral nutrition formula is often used as a supplement for patients with insufficient oral calorific intake, although the effects of additional nutrition on weight gain seem to differ depending on the components of supplementation [3,4]. The contribution of formula components to weight gain and to induction of orexigenic hormones remains unclear.

Ghrelin, a novel growth hormone (GH)-releasing peptide, was first isolated from the stomach [5] and induces a positive energy balance by stimulating food intake through

GH-independent mechanisms. Acyl-ghrelin, an active ghrelin that induces appetite through the hypothalamus, is synthesized in the stomach and inactivated as desacyl-ghrelin by deacylation. Octanoic acids are essential for acylation in the biosynthesis of acyl-ghrelin. Increased intake of octanoic acids may thus increase plasma acyl-ghrelin levels. Many reports have provided molecular analysis of ghrelin in patients with malignancy, but few have analyzed ghrelin levels in cachectic patients with CRD.

Based on the hypothesis that octanoic acids are necessary for acylation in biosynthesis of acyl-ghrelin, we investigated whether oral administration of an octanoic acid-rich formula would increase plasma acyl-ghrelin levels in cachectic patients with CRD.

Methods

Participants

We recruited 4 inpatients (2 women, 2 men; age range, 62–72 y; 2 smokers, 2 ex-smokers; duration of the illness, 2–5 y; BMI, 15.8 ± 1.7) and 19 outpatients (8 women, 11 men; age range, 62–78 y; 7 smokers, 12 ex-smokers; duration of illness, 1–10 y; BMI, 16.0 ± 2.0) with CRD. Underlying pathology was bronchiectasis in 2 and 7 patients, COPD in 1 and 7 patients, and old pulmonary tuberculosis in 1 and 5 patients, respectively. At enrolment, the following inclusion criteria were applied: i) stable respiratory disease for >6 months; and ii) cachexia with complaints of appetite loss. The following exclusion criteria were adopted: i) treatment with steroids, immunosuppressants or antibiotics prescribed within 3 months prior to the study; or ii) presence of pneumonia, cancer or asthma. Cachectic patients were defined as those with documented nonedematous and nonintentional weight loss >7.5% of previous normal weight over a period of ≤ 6 months and body mass index (BMI) <21 at entry. All patients provided written informed consent for participation and the Research Ethics Committee of Miyazaki University approved all study protocols in advance.

Study protocol

The present study set 2 protocols, as described below. First, we investigated the difference in 24-h profiles for plasma ghrelin levels with and without administration of an enteral nutrition formula rich in octanoic acids using 4 inpatients with CRD on admission. The enteral nutrition formula used here provides 3.0 g of octanoic acid triglyceride and 400 kcal per 400 ml (EN Otsuka, Naruto, Japan). The formula was prepared to provide 2.8 g/day of octanoic acid to patients when tricaprylin hydrolyzed by lipase and free octanoic acid become 100% detached. On day 1, blood samples were taken from the 4 inpatients with calorie intake limited to 1,800 kcal/day. On day 2, 400 ml of the formula was administered between breakfast and lunch in addition to meals providing 1,800 kcal. Blood samples were drawn at 07:00, 09:00, 12:00, 14:00, 17:00, 19:00 and 21:00 to identify 24-h profiles of plasma ghrelin levels. As a second trial, 400 ml/day of formula was orally administered to 19 outpatients for 2 weeks. Body weights of patients were measured at baseline and after 2 weeks of formula administration. Blood samples for these patients were taken on an empty stomach before breakfast to evaluate nutrition status and plasma ghrelin levels at baseline and after 2 weeks of formula administration. Ten age- and sex-matched healthy volunteers were

Table 1: Changes in parameters before and after 2-week once daily oral administration of octanoic acid formula to cachectic patients with chronic respiratory disease.

		Before	After	
body mass index	(kg/m^2)	16.0 ± 2.00	16.3 ± 2.00	$p < 0.05$
appetite score		40 ± 22	64 ± 27	$p < 0.05$
acyl-ghrelin	(fmol/ml)	11.0 ± 11.1	14.8 ± 7.20	$p < 0.05$
desacyl-ghrelin	(fmol/ml)	90.1 ± 52.4	90.9 ± 52.5	NS
total protein	(g/dl)	6.9 ± 0.6	7.3 ± 0.7	$p < 0.05$
albumin	(g/dl)	3.8 ± 0.4	4.0 ± 0.4	$p < 0.05$
total cholesterol	(mg/dl)	181 ± 40	184 ± 210	NS
fasting glucose	(mg/dl)	94 ± 9	91 ± 90	NS
prealbumin	(mg/dl)	15.8 ± 4.20	17.9 ± 3.90	$p < 0.05$
transferrin	(mg/dl)	198 ± 41	231 ± 570	$p < 0.05$
retinol binding protein	(mg/dl)	1.9 ± 0.4	2.3 ± 0.5	$p < 0.05$
adrenalin	(pg/ml)	63 ± 40	60 ± 21	NS
noradrenalin	(pg/ml)	852 ± 320	724 ± 298	NS
dopamine	(pg/ml)	24 ± 10	18 ± 60	NS
GH	(ng/ml)	1.2 ± 1.0	1.3 ± 1.1	NS
IGF-1	(ng/ml)	87 ± 36	98 ± 39	$p < 0.05$

Age: range 62–78 y; sex:8 women, 11 men; oral 400 ml octanoic acid formula administered once daily after breakfast in addition to food intake of 1,800 kcal

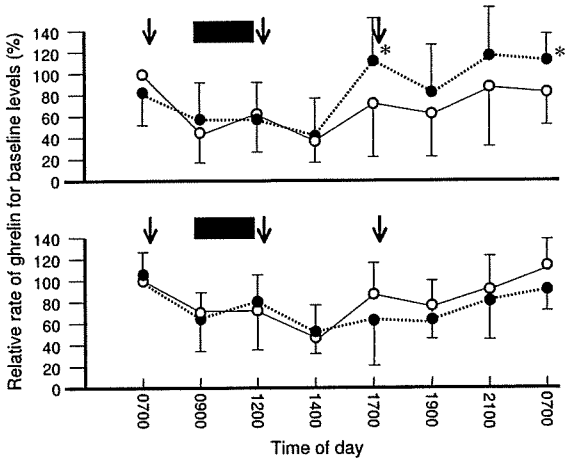

Figure 1
24-h profiles of plasma acyl-ghrelin (upper) and desacyl-ghrelin (lower). Plasma ghrelin levels peaked in the early morning and decreased after meals. Plasma ghrelin levels are shown based on a value calculated before breakfast on the morning of day 1 as 100. Plasma desacyl-ghrelin levels with formula resembled those with no formula administration, while single administration of formula between breakfast and lunch induced higher acyl-ghrelin levels before dinner, remaining high until the next morning. Open circles, levels without administration of formula; closed circles, levels with administration of formula; closed squares administration of formula. Arrows show meal-taking for inpatients with chronic respiratory disease. Data are expressed as mean ± standard deviation. *, $p < 0.05$ for ghrelin level with vs. without administration of formula.

recruited as controls to compare ghrelin levels with those in cachectic patients at baseline. Mean BMI was significantly higher in controls (20.4 ± 5.7) than in patients ($p < 0.05$).

Blood sampling and assay

Blood samplings were performed at baseline and during the week after the end of therapy to measure levels of total protein, albumin, glucose, total cholesterol, triglycerides and rapid-turnover proteins. Blood samples were taken from an antecubital vein after 30-min bed rest in the morning following an overnight fast. Plasma acyl-ghrelin and desacyl-ghrelin levels were measured by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Iatron, Tokyo, Japan). Immunoradiometric assays were used to measure levels of serum GH (Ab Bead HGH Eiken; Eiken Chemical, Tokyo, Japan) and insulin-like growth factor (IGF)-1 (Somatomedin CII Bayer; Bayer Medical, Tokyo, Japan).

Appetite assessment

Appetite in patients was quantified using the Edmonton Symptom Assessment Scale [6], which uses a 100-mm visual analog scale for appetite. Before and after 2-week administration of formula, appetite in patients was assessed before breakfast between 08:00 and 09:00.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparison of ghrelin levels between the 2 groups was analyzed using the Mann-Whitney U test. Changes in parameters between the 2 groups were analyzed using the Wilcoxon signed-rank test. Values of $p < 0.05$ were taken to indicate statistical significance.

Results

Plasma ghrelin levels in patients with chronic pulmonary disease at study entry

Plasma acyl-ghrelin and desacyl-ghrelin levels were 11.0 ± 11.1 fmol/ml and 90.1 ± 52.4 fmol/ml, respectively, in the 19 outpatients with CRD (Table 1). Acyl-ghrelin levels trended to be lower and desacyl-ghrelin levels to be higher in patients than in controls (patients: 15.1 ± 12.9 fmol/ml; range, 4.0–42.5 fmol/ml and controls: 68.7 ± 62.0 fmol/ml; range, 20.5–197.5 fmol/ml, respectively), although no significant differences were identified. The sum of both forms of ghrelin was higher in patients (101.1 ± 58.8 fmol/ml) than in controls (83.7 ± 74.3 fmol/ml). The ratio of plasma acyl-ghrelin to desacyl-ghrelin was lower in patients (0.15 ± 0.16) than in controls (0.24 ± 0.10).

Ghrelin 24-h profiles with and without single administration of formula

Plasma ghrelin levels peaked in the early morning and decreased after meals, supporting the findings of previous reports (Figure 1). Plasma desacyl-ghrelin levels with formula resembled those with no formula administration, while single administration of 400 ml of formula between breakfast and lunch induced higher acyl-ghrelin levels before dinner, remaining high until the next morning.

Effect of 2-week administration of formula on plasma ghrelin, appetite, weight, nutrition status and hormone levels

Significant increases were seen in levels of plasma acyl-ghrelin, appetite score and body weight, but not desacyl-ghrelin. Levels of serum total protein, albumin and rapid turnover proteins increased after two-week administration of formula. No correlations were identified between the increases in acyl-ghrelin levels and weight or nutrition parameters. Two-week administration of formula did not alter fasting glucose, total cholesterol, triglyceride, catecholamines or GH levels, but induced an increase in serum IGF-1 levels.

Discussion

This is the first paper showing a molecular analysis of plasma ghrelin in cachectic patients with CRD. Ghrelin

profiles during the study showed that the total level of acyl-ghrelin and desacyl-ghrelin was high, but the ratio of plasma acyl-ghrelin to desacyl-ghrelin was low in cachectic CRD patients. High levels of ghrelin in cachectic patients have been suggested to maintain energy balance to prevent weight loss, consistent with previously studies reporting an inverse correlation between BMI and plasma ghrelin levels [7,8]. Acylation is necessary for ghrelin to induce appetite and desacyl-ghrelin is likely to inhibit appetite in mice [9], suggesting that the ratio of acylated ghrelin to desacyl-ghrelin may be important in determining the orexigenic effects of ghrelin.

The present study showed that administration of formula containing high levels of octanoic acids increased plasma acyl-ghrelin levels along with weight in patients with CRD. The study was designed for outpatients and exact food intake including formula during the 2-week period was not measured. Weight gain may have been due to the additional energy provided by the formula in addition to regular meals. In the present study, 2-week administration of the formula induced an increase in both weight and plasma acyl-ghrelin levels, suggesting that weight gain was associated with increases in acyl-ghrelin and the orexigenic effect was due to decreased plasma ghrelin levels when the patients displayed weight increases.

Additional induction of acyl-ghrelin induced a significant increase in IGF-1 levels. The concentration of circulating IGF-1 declines with age [10] and this hormone is involved in physiological changes of aging such as increased cardiovascular risk, reduced muscle mass and strength, reduced exercise tolerance and impaired quality of life [11]. IGF-1 stimulates osteoblast proliferation as well as osteoclast differentiation to inhibit osteopenia [12]. CRD with airflow obstruction has been shown to represent a causative risk for osteoporosis [13], so elevation of IGF-1 levels may be particularly useful for elderly individuals with CRD.

In conclusion, formula containing octanoic acids increased body weight and plasma acyl-ghrelin levels. This is the first trial showing a change in orexigenic hormone among patients receiving nourishment treatment. The present results seem likely to contribute to nutritional management in patients with cachectic diseases.

Abbreviations

CRD: chronic respiratory disease; GH: growth hormone; IGF-1: insulin-like growth factor-1; BMI: body mass index.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JA participated in study design, data analysis and manuscript preparation. NM participated in data collection and data analysis. MN participated in manuscript preparation and editing. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Ms. S. Tajiri for her excellent technical assistance. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Alp E, Güven M, Yildiz O, Aygen B, Voss A, Doganay M: **Incidence, risk factors and mortality of nosocomial pneumonia in intensive care units: a prospective study.** *Ann Clin Microbiol Antimicrob* 2004, **3**:17.
2. Chailleux E, Laaban JP, Veale D: **Prognostic value of nutritional depletion in patients with COPD treated by long-term oxygen therapy: data from the ANTADIR observatory.** *Chest* 2003, **123**:1460-1466.
3. Creutzberg EC, Schols AM, Weling-Scheepers CA, Buurman WA, Wouters EF: **Characterization of nonresponse to high caloric oral nutritional therapy in depleted patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 2000, **161**:745-752.
4. Matsuyama W, Mitsuyama H, Watanabe M, Oonakahara K, Higashimoto I, Osame M, Arimura K: **Effects of omega-3 polyunsaturated fatty acids on inflammatory markers in COPD.** *Chest* 2005, **128**:3817-3827.
5. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: **Ghrelin is a growth-hormone-releasing acylated peptide from stomach.** *Nature* 1999, **402**:656-660.
6. Bruera E, Kuehn N, Miller MJ, Selmser P, Macmillan K: **The Edmonton Symptom Assessment System (ESAS): a simple method for the assessment of palliative care patients.** *J Palliat Care* 1991, **7**:6-9.
7. Itoh T, Nagaya N, Yoshikawa M, Fukuoka A, Takenaka H, Shimizu Y, Haruta Y, Oya H, Yamagishi M, Hosoda H, Kangawa K, Kimura H: **Elevated plasma ghrelin level in underweight patients with chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 2004, **170**:879-882.
8. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura : **Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion.** *J Clin Endocrinol Metab* 2002, **87**:240-244.
9. Asakawa A, Inui A, Kaga T, Katsuura G, Fujimiya M, Fujino MA, *et al.*: *Gut* 2003, **52**:947-52.
10. Landin-Wilhelmsen K, Wilhelmsen L, Lappas G, Rosén T, Lindstedt G, Lundberg PA, Bengtsson BA: **Serum insulin-like growth factor I in a random population sample of men and women: relation to age, sex, smoking habits, coffee consumption and physical activity, blood pressure and concentrations of plasma lipids, fibrinogen, parathyroid hormone and osteocalcin.** *Clin Endocrinol (Oxf)* 1994, **41**:351-357.
11. Venken K, Movérare-Skrtic S, Kopchick JJ, Coschigano KT, Ohlsson C, Boonen S, Bouillon R, Vanderschueren D: **Impact of androgens, growth hormone, and IGF-I on bone and muscle in male mice during puberty.** *J Bone Miner Res* 2007, **22**:72-82.
12. Rucker D, Ezzat S, Diamandi A, Khosravi J, Hanley DA: **IGF-I and testosterone levels as predictors of bone mineral density in healthy, community-dwelling men.** *Clin Endocrinol (Oxf)* 2004, **60**:491-499.
13. Sabit R, Bolton CE, Edwards PH, Pettit RJ, Evans WD, McEniery CM, Wilkinson IB, Cockcroft JR, Shale DJ: **Arterial stiffness and osteoporosis in chronic obstructive pulmonary disease.** *Am J Respir Crit Care Med* 2007, **175**:1259-1265.

Inflamm. res. 58 (2009) 192–197
1023-3830/09/0400192-6
DOI 10.1007/s00011-008-8120-8

Inflammation Research

Concentrations of α- and β-defensins in plasma of patients with inflammatory bowel disease

N. Yamaguchi[1], H. Isomoto[1], H. Mukae[1], H. Ishimoto[1], K. Ohnita[1], S. Shikuwa[1], Y. Mizuta[1], M. Nakazato[2], S. Kohno[1]

[1] Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan,
Fax +81-95-849-7285, e-mail hajimei2002@yahoo.co.jp
[2] Third Department of Internal Medicine, Miyazaki University School of Medicine, Miyazaki, Japan

Received 2 June 2008; returned for revision 20 June 2008; received from final revision 25 June 2008; accepted by C. Kasserra 19 August 2008

Published Online First 31 January 2009

Abstract. *Background:* Impaired production/release of defensins, representative endogenous antimicrobial peptides, is associated with the pathogenesis of inflammatory bowel disease (IBD). *Material and methods:* Employing in house radioimmunoassay, we examined concentrations of the major forms α-defensins, human neutrophil peptides (HNP) 1–3 and human β-defensin (HBD)-2 in plasma of 55 IBD patients consisting of 29 patients with ulcerative colitis (UC) and 26 with Crohn's disease (CD) and 57 controls.
Results: The circulating HNP 1–3, but not HBD-2, levels in IBD patients were significantly higher than those in controls. Plasma HNP 1–3 concentrations in CD patients significantly correlated with Crohn's disease activity index, peripheral white blood cell counts, serum CRP values and TNF-α levels.
Conclusions: Elevation of circulating α-defensins levels is suggestive of their physiopathological roles in IBD. Plasma HNP 1–3 concentrations may be an indicator for CD activity and their association with CRP and TNF-α supports a possible association with the inflammatory process.

Key words: human neutrophil peptides 1–3 – human β-defensin-2 – inflammatory bowel disease – ulcerative colitis – Crohn's disease.

Introduction

Recently, endogenous antimicrobial peptides have been identified as key elements of innate host defence against infection [1–3]. Defensins, single chain cationic peptides with molecular weight ranging from 3000 to 4500, are one of the most extensively studied classes of such naturally occurring antibiotics [1–3]. Human defensins are divided into α- and β-defensins, based on the arrangements of three intramolecular disulphide bridges [1–3]. Among six members of human α-defensins identified so far, human neutrophil peptides 1–4 (HNP-1, HNP-2, HNP-3 and HNP-4) are localized in azurophilic granules of neutrophils. HNP 1–3 are very similar and different only in a single N-terminal amino acid, whereas HNP-4 shares only 32% amino acid sequence homology to HNP 1–3 [1–3]. The other two, named human defensin-5 (HD-5) and HD-6, are present in intestinal Paneth's cells [1, 2]. On the other hand, the four human β-defensins including human β-defensin (HBD)-1 and HBD-2 are primarily produced by epithelia at mucosal sites [1–3]. They exhibit a wide variety of microbicidal activities against Gram-positive and -negative bacteria, mycobacteria, fungi and certain enveloped viruses [1–3]. In fact, *in vivo* studies have shown elevated concentrations of these defensins in blood and body fluids from patients infected with various microorganisms [1–3].

Chronic inflammatory bowel disease (IBD) is a multifactorial disorder that is characterized by inflammation specific to the gastrointestinal tract, which results in intestinal malabsorption, immune defense abnormalities, and an exaggerated inflammatory response [4, 5]. Various immune and inflammatory cells, such as lymphocytes, macrophages, and dendritic cells, play important roles in the development and progression of IBD [6–8]. Based on the clinical features and histopathology, IBD is classified into two major entities, ulcerative colitis (UC) and Crohn's disease (CD) [4, 5]. Although the precise etiology of IBD remains unknown, both several environmental factors, such as dietary components and microorganisms, and genetic factors may contribute to the occurrence of IBD [9–11]. Intestinal microbiota are implied in the pathological inflammation and may mediate both innate and adaptive responses underlying the chronic inflammation [9–11].

Correspondence to: H. Isomoto

Table 1. The characteristics of subjects studied

Characteristics	Patients with		Controls
	UC	CD	
Number	29	26	57
Age (years)	42.4 ± 15.8	32.8±9.7	36.6 ± 10.3
Age range (yrs)	16–78	15–56	17–56
Male/female	14/15	17/9	30/27
Extent of UC			
Proctitis	3		
Left sided UC	11		
Pancolitis	15		
Location of CD			
Ileal		3	
Colonic		3	
Ileocolonic		20	
Disease severity of UC			
Mild	11		
Moderate	15		
Severe	3		
Disease activity of CD			
Active		8	
Inactive		18	
Behavior of CD			
Stricture		17	
Penetration		8	
Perianal diseases		12	

UC, ulcerative colitis, CD, Crohn's disease

Although defensins were first identified as antimicrobial peptides, recent evidence suggest that they can act with host immune cells, thereby playing important roles in both the innate and adaptive immune responses against infection agents [1–3]. Recent study has shown that variants in HBD1 gene are inversely associated with serological markers, antiglycan antibodies in CD patients, supporting the role of the defensins in the pathogenesis [12]. We have developed a sensitive, specific radioimmunoassay (RIA) for HNP 1–3 and HBD-1-2, major forms of α- and β-defensins, respectively [13, 14]. Employing this assay system, we measured their concentrations in plasma of patients with UC or CD. In addition, we assessed the relationship between the concentrations of α- and β-defensins and various clinical characteristics of each IBD.

Materials and methods

Patients and sampling

The study subjects comprised 29 patients with UC, 26 patients with CD, and 57 controls. The characteristics of subjects are shown in Table 1. All participants were Japanese. The diagnosis of IBD was made on the basis of the endoscopic, radiological, histological, and clinical criteria provided by the WHO Council for International Organizations of Medical Sciences and the International Organization for the Study of Inflammatory Bowel Disease [15–17]. Patients with infectious diseases were excluded from the subjects in this study. Patients with UC were classified into subgroups according to extension of lesions (proctitis, left-sided colitis, or pancolitis) and disease severity (mild, moderate, or severe) (Table 1). Likewise, patient with CD were divided into subgroups according to localization of lesions (ileal, ileocolonic, or colonic), behaviour of disease (stricture and penetration), and disease activity (active or inactive) (Table 1). These were stratified in accordance with Montreal classification [18] with slight modification. A clinical activity index (CAI) [19] and Crohn's disease activity index (CDAI) [20] were scored for UC and CD.

Blood samples were withdrawn, transferred into tubes containing ethylenediaminetetraacetic acid (EDTA)-2Na and aprotinin, centrifuged, plasma separated, and then stored at –80 °C until assay.

Measurement of HNP 1–3 and HBD-2 levels in plasma

Concentrations of the α- and β-defensins in plasma were measured by RIA established in our laboratory [13, 14]. Briefly, full-length HNP-1 and HBD–2 were synthesized using a peptide synthesizer (model 430, Applied Biosystems, Foster City, CA) and purified by reverse phase high performance liquid chromatography (RP-HPLC). The synthetic peptides were used for immunizing New Zealand white rabbits by multiple intracutaneous and subcutaneous injections. They were radioiodinated and the ^{125}I-labelled peptides were purified by RP-HPLC on a TSK ODS 120A column (Tosoh Co., Tokyo). A diluted sample or standard peptide solution (100l) was incubated for 24 hours with each 100l antiserum diluent. The final dilutions were 1: 21000 and 1: 4200000 for HNP-1 and HBD-2, respectively. The ^{125}I-labelled solution (16000cpm in 100L) was added and the mixture was incubated again for another 24 h. Normal rabbit serum and anti-rabbit IgG goat serum were then added and stored for 16 hours. Bound and free ligands were separated by centrifugation. All procedures were performed at 4 °C and duplicate assays were carried out. Each 10L of plasma was used to determine the levels of defensins. The antiserum for HNP-1 recognized HNP-2 and HNP-3 equally on a molar basis, and thus the RIA data were expressed as the sum of HNP 1–3 [14]. The intra-assay and inter-assay coefficients of variation were <10 % in all RIA analyses [13, 14].

Circulating tumor necrosis factor α and interleukin 8 concentrations

Measurement of tumor necrosis factor α (TNFα) and interleukin 8 (IL-8) in plasma was performed using commercially available assay kit (Research and Diagnostics, Minneapolis, MN), which employs the quantitative immunometric sandwich enzyme immunoassay technique. These assays were performed in duplicate according to the instructions provided by the manufacturer. In our study, inter- and intra-assay variabilities were <10 %, respectively.

Statistical analysis and ethical considerations

Statistical analyses were performed using Fisher's exact, χ^2, Student's *t*, Mann-Whitney U, Kruskal-Wallis, Spearman rank and Wilcoxon signed ranks tests, as appropriate. A p value of less than 0.05 was accepted as statistically significant. Data were expressed as mean ± SD.

All examinations were conducted according to the Good Clinical Practice and the Declaration of Helsinki, and approved by the University Ethics Committees. All samples were obtained with written informed consent of the patients prior to their inclusion in the study.

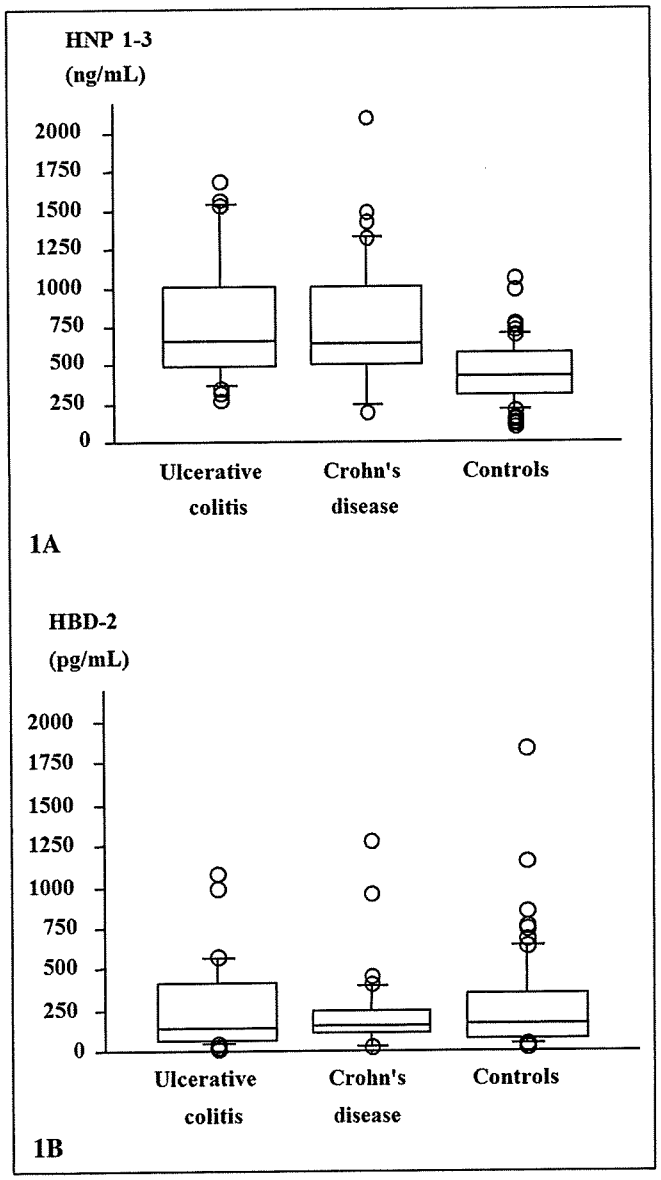

Fig. 1 A. Concentrations of human neutrophil peptides 1–3 (HNP 1–3), **B:** Concentrations of human β-defensin 2 (HBD-2) in plasma of patients with ulcerative colitis and Crohn's disease and controls.

Results

Concentrations of the α- and β-defensins in plasma of IBD patients

The HNP 1–3 levels in plasma significantly differed among UC and CD patients and controls (Fig. 1A). The circulating HNP 1–3 levels in the CD and UC group were significantly higher than the control group ($p<0.0001$ for each). There was no significant difference between the UC and CD group (Fig. 1A). There was no significant difference in HBD-2 levels in plasma among UC and CD patients and controls (Fig. 1B). There were no significant differences in plasma HNP 1–3 and HBD-2 concentrations among the subgroups both for CD and UC patients (Table 2). There was no significant correlation between HNP 1–3 and HBD-2 levels in plasma with a correlation coefficient being 0.118 in all the IBD patients. The correlation coefficients were 0.043 and 0.341 in the UC and CD group, respectively (not significant for each).

Clinical activity of IBD and concentrations of the α- and β-defensins in plasma

Plasma HNP 1–3 concentrations did not correlate with CAI (correlation coefficient=0.059), whereas the correlation coefficient with CDAI was 0.387 ($p<0.05$) (Fig. 2A). There were no significant correlations between HBD-2 levels in plasma and CAI or CDAI (-0.103 or 0.300, respectively).

Plasma HNP 1–3 and HBD-2 levels and laboratory data

HNP 1–3 levels in plasma significantly correlated with peripheral white blood cell counts with a correlation coefficient being 0.274 ($p<0.05$) in all the IBD patients. In CD patients, the correlation coefficient between plasma HNP 1–3 concentrations and the white blood cell counts was 0.501 ($p<0.01$) (Fig. 2B), whereas it was 0.107 (not significant) in the UC group. Moreover, circulating HNP 1–3 levels significantly correlated with peripheral blood neutrophil counts in CD (correlation coefficient=0.495, $p<0.01$) but not UC group (correlation coefficient=0.039, not significant) (data not shown). There was a significant correlation between HNP 1–3 levels in plasma and serum C-reactive protein (CRP) levels in the CD group (correlation coefficient=0.446, $p<0.05$, Fig. 2C), whereas it was 0.016 (not significant) in the UC group. On the contrary, HBD-2 levels in plasma did not correlate with WBC or serum CRP levels, irrespective of the IBD subtype. There was no significant association of the circulating α- or β-defensins levels with the other laboratory data.

The proinflammatory cytokines and the α- and β-defensins levels in plasma

There were a significant correlation between TNF-α and HNP 1–3 levels in plasma (correlation coefficient=0.425, $p<0.005$) in all the IBD patients. The correlation coefficients were 0.437 ($p<0.05$) and 0.411 ($p<0.05$) in the UC and CD group, respectively (Fig. 2D). On the other hand, there was no association between TNF-α and HBD-2 levels in plasma, irrespective of the IBD subtype. Again, plasma IL-8 levels were below the detectable levels (<8 pg/mL) in all the IBD patients.

Discussion

We found that the concentrations of HNP 1–3, but not HBD-2, in plasma were significantly elevated in IBD patients compared to controls employing the aforementioned sensitive RIA system [13, 14]. Despite a lack of information on circulating levels of the α- as well as β-defensins in IBD, their local expression has been extensively studied in intestinal mu-

Table 2. Human neutrophil peptides 1-3 and β-defensin 2 concentrations in plasma with respect to clinical characteristics of Ulcerative colitis (UC) and Crohn's disease (CD)

Characteristics	Plasma concentrations	
	Human neutrophil peptides 1-3 (ng/mL)	Human β-defensin 2 (pg/mL)
Extent of UC		
Proctitis	458.3 ±139.5	290.3 ±250.2
Left sided UC	740.2 ±320.3	233.9 ±169.5
Pancolitis	902.2 ±463.0	226.3 ±200.9
Location of CD		
Ileal	889.0 ±207.8	166.0 ±82.4
Colonic	710.1 ±135.0	152.9 ±50.8
Ileocolonic	930.0 ±448.4	191.1 ±124.2
Disease severity of UC		
Mild	612.5±281.2	264.9 ±187.3
Moderate	941.9 ±468.1	219.6 ±178.2
Severe	728.0±156.0	210.3±298.1
Disease activity of CD		
Active	1040.8±543.1	199.0±143.1
Inactive	837.2±323.2	177.0±100.1
Behavior of CD		
Stricture		
Present/Absent	910.1±452.3/880.5±314.5	195.7±109.8/161.3±120.8
Penetration		
Present/Absent	1130.8±490.9/797.2±313.5	212.9±132.3/170.9±104.1
Perianal diseases		
Present/Absent	1029.2±298.1/789.0±283.6	206.0±137.4/164.8±86.8

cosa of the patients [21–25]. This study is novel, as the data on circulating defensins or HNP are not readily available.

There were no differences in plasma HNP 1–3 concentrations between CD and UC patients. However, the mechanisms underlying the elevation of the α-defensins appeared to be different in the two IBD entities. There were significant association of circulating HNP 1–3 levels with peripheral white blood cell and neutrophil counts in CD but not UC patients. The synthesis of HNP is restricted to cells of neutrophil lineage [26]. The majority of pro-HNP are processed in neutrophil precursor cells within the bone marrow, and then to mature HNP in peripheral neutrophils [14]. HNP 1–3 constitute 30 to 50% of total protein content of azurophilic granules in neutrophils, where they are the most abundant antimicrobial peptides, and contribute to the oxygen-independent killing of microorganisms [1, 2]. Thus, the source of the α-defensins elevated in plasma of CD patients can be from circulating neutrophils, albeit the other cell types such as dendritic cells are known to secrete HNP 1–3 [27]. In this regard, immunohistochemical analysis revealed that surface enterocytes strongly positive for HNP 1–3 as well as lysozyme were seen in inflamed colonic mucosa [22], suggesting that the elevated HNP 1–3 may be in part derived from colonic epithelial cells in UC patients. Alternatively, some portion of the HNP may be released from infiltrating neutrophils within the affected colon into circulation.

In CD patients, plasma HNP 1–3 concentrations significantly correlated with CDAI as well as serum CRP values and plasma concentrations of TNF-α, a representative proinflammatory cytokine. The circulating HNP 1–3 levels can be as a biomarker for the disease activity of CD, reflecting the degree of inflammatory responses. HNP are known to be potent chemotaxins for macrophages, dendritic cells and T lymphocytes [28, 29]. They stimulate IL-8 synthesis by epithelial cells [30] and control IL-1β processing and release in monocytes [31]. These immunomodulatory properties of α-defensins are suggestive of their implication in the CD pathogenesis. On the other hand, they did not correlate with CAI or CRP values but TNF-α levels in UC. Upon stimulation by TNF-α and IL-1β, CaCo-2, a human enterocyte-derived cell line, is able to express HD-5, HD-6, HBD-1 and HBD-2 [21, 32]. Colonic synthesis of HBD-2-4 is dependent on NF (nuclear factor) B activation by infectious agents and/or proinflammatory cytokines including TNF-α [33, 34]. Although the inductive effect of TNF-α on HNP remains to

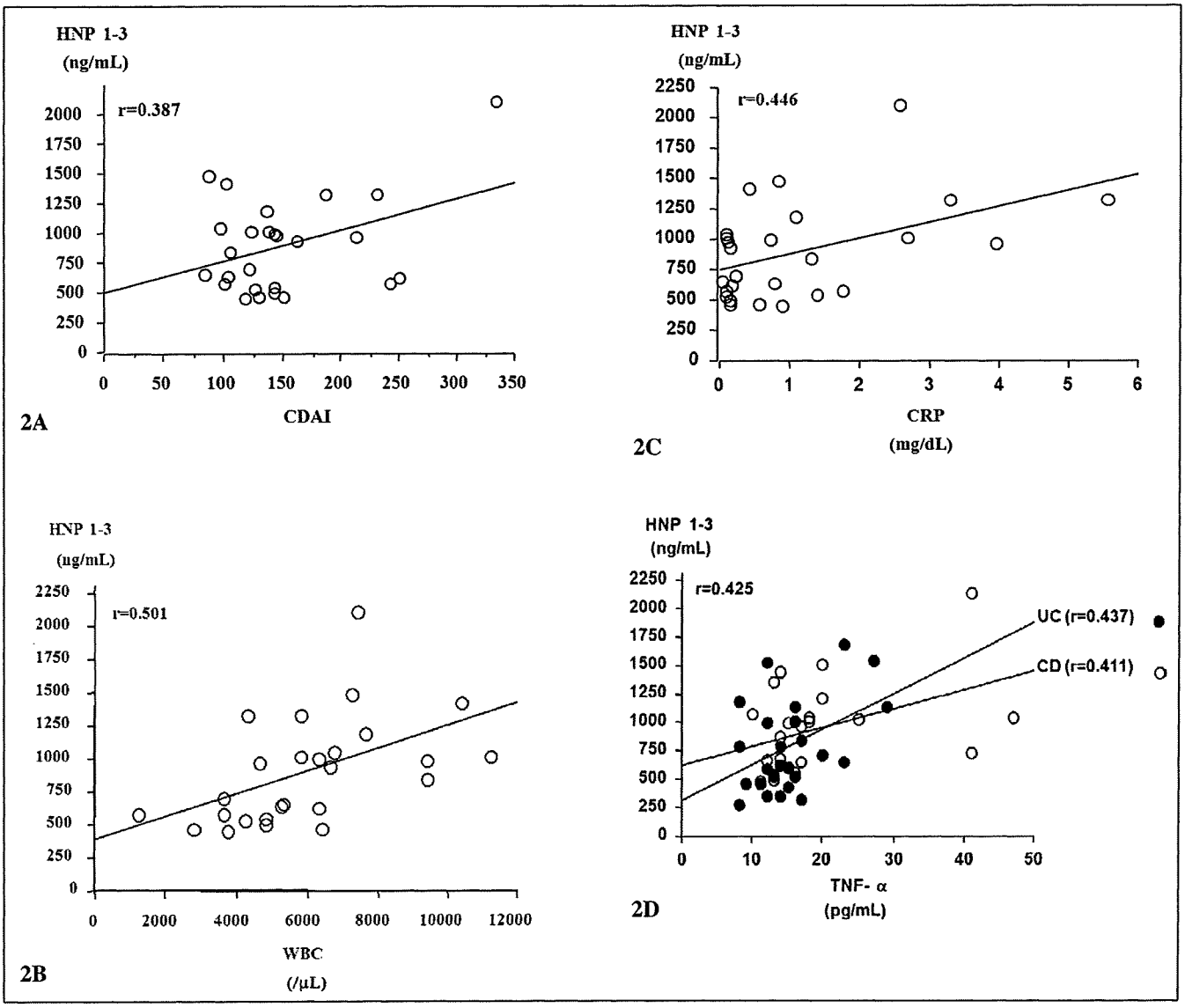

Fig. 2 A. Relationship between concentrations of HNP 1–3 in plasma of Crohn's disease patients and Crohn's disease activity index (CDAI). **B:** Relationship between concentrations of HNP 1–3 in plasma of CD patients and peripheral white blood cell counts (WBC). **C:** Relationship between concentrations of HNP 1–3 in plasma of CD patients and serum C-reactive protein (CRP). **D:** Relationship between concentrations of HNP 1–3 in plasma of UC and CD patients and circulating tumor necrosis factor α (TNF-α) levels.

be determined, such proinflammatory mediators might promote the α-defensins production and their release from colonic mucosa into blood.

Plasma HBD-2 concentrations in IBD patients were comparable to those in controls. Moreover, there were no differences in its circulating levels with respect to clinical characteristics irrespective of IBD subgroups. These results suggest that plasma measurement of HBD-2 concentrations is unlikely to be informative in the clinical settings of IBD. Nevertheless, upregulation of HBD-2 is evidently observed in colonic epithelial cells of the inflamed mucosa of UC [22, 34]. In this regard, we reported that HBD-2 concentrations in gastric juice of patients with H pylori-associated gastritis were significantly higher than those of uninfected subjects, whereas H pylori status did not have a significant impact on plasma HBD-2 levels [35]. In turn, CD of the colon is associated with a low-gene copy number polymorphism of the human β-defensins locus resulting in an attenuated β-defensins induction in enterocytes [36]. The HBD-2 product may be released mainly into the gastrointestinal lumen but not into blood stream even in cases of the digestive inflammatory disorders.

In conclusion, we for the first time demonstrated significant elevation of plasma HNP 1–3 concentrations in IBD. In CD patients, the association of HNP 1–3 levels with CDAI along with white blood cell counts, CRP values and TNF-α in blood involves α-defensins in an informative biomarker for the disease activity, possibly through their functions as immune and inflammatory mediators. However, these conclusions should be interpreted within the limitation of the small sample size. Clearly, further studies in a larger numbers of IBD cohorts are needed.

References

[1] van Wetering S, Sterk PJ, Rabe KF Hiemstra PS. Defensins: Key players or bystanders in infection, injury, and repair in the lung. J Allergy Clin Immunol 1999; 104: 1131–8.

[2] Chertov O, Yang D, Howard OM, Oppenheim JJ. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. Immunol Rev 2000; 177: 68–78.

[3] Bals R. Epithelial antimicrobial peptides in host defense against infection. Respir Res 2000; 1: 141–150

[4] Danese S, Fiocchi C. Etiopathogenesis of inflammatory bowel diseases. World J Gastroenterol 2006; 12: 4807–12.

[5] Farrell RJ, Peppercorn MA. Ulcerative colitis. Lancet 2002; 359: 331–40.

[6] Cho JH, Nicolae DL, Ramos R, Fields CT, Rabenau K, Corradino S et al. Linkage and linkage disequilibrium in chromosome band 1p36 in American Chaldeans with inflammatory bowel disease. Hum Mol Genet 2002; 9: 1425–32.

[7] Taylor KD, Yang H, Rotter JI. Inflammatory bowel disease, II, Gene mapping. Mol Genet Metab 2001; 74: 22–44.

[8] Mizoguchi A, Mizoguchi E, Bhan AK. Immune networks in animal models of inflammatory bowel disease. Inflamm Bowel Dis 2003; 9: 246–59.

[9] Yang H, Taylor KD, Rotter JI. Inflammatory bowel disease, I, Genetic epidemiology. Mol Genet Metab 2001; 74: 1–21.

[10] Watts DA, Satsangi J. The genetic jigsaw of inflammatory bowel disease, Gut 2002; Suppl 3: 31–6.

[11] Bonen DK, Cho JH. The Genetics of Inflammatory Bowel Disease, Gastroenterology 2003; 124: 521–36.

[12] Lakatos PL, Altorjay I, Mándi Y, Lakatos L, Tumpek J, Kovacs A et al. Interaction between seroreactivity to microbial antigens and genetics in Crohn's disease: is there a role for defensins? Tissue Antigens 2008; 71: 552–9.

[13] Hiratsuka T, Nakazato M, Date Y, Ashitani J, Minematsu T, Chino N et al. Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. Biochem Biophys Res Commun 1998: 249: 943–7.

[14] Nakazato M, Shiomi K, Date Y, Matsukura S, Kangawa K, Minamino N et al. Isolation and sequence determinants of 6- and 8-kDa precursors of human neutrophil peptides from bone marrow, plasma and peripheral blood neutrophils. Biochem Biophys Res Commun 1995; 211: 1053–62.

[15] Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol 1996; Suppl 170: 2–6.

[16] Podolsky DK. Inflammatory bowel disease (1). N Engl J Med 1991; 325: 928–37.

[17] Podolsky DK. Inflammatory bowel disease (2). N Engl J Med 1991; 325: 1008–16.

[18] Satsangi J, Silverberg MS, Vermeire S Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006; 55: 749–53.

[19] Rachmilewitz D. Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis: a randomised trial. BMJ 1989; 14: 82–6.

[20] Best WR, Becktel JM, Singleton JW, Singleton JW. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology 1976; 70: 439–44.

[21] Shi J. Defensins and Paneth cells in inflammatory bowel disease. Inflamm Bowel Dis 2007; 13: 1284–92.

[22] Fahlgren A, Hammarstrom S, Danielsson A, Hammarström ML. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. Clin Exp Immunol 2003; 131: 90–101.

[23] Wehkamp J, Schmid M, Fellermann K, Stange EF. Defensin deficiency, intestinal microbes, and the clinical phenotypes of Crohn's disease. J Leukoc Biol 2005; 77: 460–5.

[24] Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, et al. Reduced Paneth cell a-defensin in ileal Crohn's disease. Proc Natl Acad Sci USA 2005; 102: 18129–34.

[25] Peyrin-Biroulet L, Vignal C, Dessein R, Simonet M, Desreumaux P, Chamaillard M. NODs in defence: from vulnerable antimicrobial peptides to chronic inflammation. TRENDS in Microbiology 2006; 14: 432–8.

[26] Date Y, Nakazato M, Shiomi K, Toshimori H, Kangawa K, Matsuo H et al. Localization of human neutrophil peptide (HNP) and its messenger RNA in neutrophil series. Ann Hematol 1994; 69: 73–7.

[27] Rodríguez-García M, Oliva H, Climent N García F, Gatell JM, Gallart T. Human immature monocyte-derived dendritic cells produce and secrete alpha-defensins 1-3. J Leukoc Biol 2007; 82: 1143–6.

[28] Yang D, Chen Q, Chertov O, Oppenheim JJ. Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. J Leukoc Biol 2000; 68: 9–14.

[29] Grigat J, Soruri A, Forssmann U, Riggert J, Zwirner J. Chemoattraction of macrophages, T lymphocytes, and mast cells is evolutionarilly concerved within the human alpha-defensin family. J Immunol 2007; 179: 3958–65.

[30] Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am J Physiol 1997; 272: 888–96.

[31] Shi J, Aono S, Lu W, Ouellette AJ, Hu X, Ji Y, et al. A novel role of defensins in intestinal homeostasis: regulation of IL-1β secretion. J Immunol 2007; 179: 1245–53.

[32] Wehkamp J, Schwind B, Herrlinger KR, Baxmann S, Schmidt K, Duchrow M, et al. Innate immunity and colonic inflammation: enhanced expression of alpha-defensins. Dig Dis Sci 2002; 47: 1349–55.

[33] Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003; 3: 710–20.

[34] O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, et al. Expression and regulation of the human β-defensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 1999; 163: 6718–24.

[35] Isomoto H, Mukae H, Ishimoto H, Nishi Y, Wen CY, Wada A, et al. High concentrations of human beta-defensin 2 in gastric juice of patients with *Helicobacter pylori* infection. World J Gastroenterol 2005; 11: 4782–7.

[36] Fellermann K, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, Bevins CL, et al. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. Am J Hum Genet 2006; 79: 439–48.

Cell. Mol. Life Sci. 66 (2009) 1939–1945
1420-682X/09/111939-7
DOI 10.1007/s00018-009-8796-0

Cellular and Molecular Life Sciences

Review

Neuroendocrine regulatory peptide-1 and -2: Novel bioactive peptides processed from VGF

K. Toshinai and M. Nakazato*

Neurology, Respirology, Endocrinology and Metabolism, Division of Internal Medicine, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889–1692 (Japan), Fax: +81-985-85-1869,
e-mail: nakazato@med.miyazaki-u.ac.jp

Received 17 December 2008; accepted 13 January 2009
Online First 6 February 2009

Abstract. Neuroendocrine regulatory peptides (NERP)-1 and NERP-2 are derived from distinct regions of VGF, a neurosecretory protein that was originally identified as a product of a nerve growth factor-responsive gene in rat PC12 cells. The amino acid length of human NERP-1 is 26, and that of rat NERP-1 is 25. Human and rat NERP-2 are both 38 amino acid peptides. NERPs colocalize with vasopressin in the storage granules of the paraventricular and supraoptic nuclei in the hypothalamus of both rats and humans. Administration of NERPs suppresses hypertonic saline- or angiotensin II-induced vasopressin release from the hypothalamus and pituitary. Thus, VGF is a precursor of multiple bioactive peptides with diverse neuroendocrine functions, and NERPs are novel hypothalamic peptides involved in the control of body fluid homeostasis by regulating vasopressin release.

Keywords. Neuroendocrine regulatory peptide, vasopressin, hypothalamus, VGF, processing, feeding regulation.

Introduction

The identification of new bioactive peptides is an important step towards elucidation of novel biological systems in the body and the development of innovative drugs. A large array of peptide hormones and neuropeptides function as cell-cell signaling molecules and mediate a variety of physiological processes. The hypothalamus, which occupies the ventral half of the diencephalon and lies immediately above the pituitary gland, functions as an essential interface between endocrine, autonomic, and somatomotor systems [1]. It regulates the cardiovascular system, thermoregulatory responses, and the abdominal viscera, as well as defensive-aggressive behavior, feeding behaviors, and sexual and maternal behaviors. Many neuropeptides serve as signaling molecules in hypothalamic control mechanisms linking the brain and peripheral organs. The majority of peptide receptors are G protein-coupled receptors (GPCRs), but the ligands for these GPCRs have not all been identified. An endogenous ligand screen using cell lines that artificially express orphan GPCRs, together with genetic engineering techniques, has expanded our understanding of novel cell-cell signaling systems in the hypothalamus. Some hypothalamic neuropeptides, such as orexins [2], ghrelin [3], and neuropeptide W [4] have been identified as ligands for orphan GPCRs. However, although an increasing number of mammalian genomes have been sequenced, the discovery of new bioactive peptides has not increased at

* Corresponding author.

the expected pace, mostly due to the lack of *in vivo* data on bioactive peptides.

Bioactive peptides are cleaved from their precursor proteins via limited cleavage and often must undergo post-translational modifications to become biologically active [5, 6]. Recently, a comprehensive analysis of all secretory peptides produced by human medullary thyroid carcinoma TT cells identified 230 peptides [7]. Some derive from calcitonin, a peptide functioning in skeletal conservation and fertilization [8–10], others from calcitonin gene-related peptide (CGRP), a potent dilator of blood vessels [11]. Still others derive from chromogranin, secretogranin, or other known peptide hormone precursors [12]. Only 10% of the 230 peptides were derived from proteins annotated as non-secretory peptides in the public database. Nineteen peptides with C-terminal amidation, the post-translational modification most frequently observed in bioactive peptides [13], were identified, with 15 corresponding to entire or partial sequences of calcitonin and CGRP [7]. Of particular note were two novel amidated peptides with monoisotopic masses of 2677.4 and 4062.2 and shorter fragments with masses of 2521.4 and 3405.2. We designated these neuroendocrine regulatory peptides (NERP)-1 and -2 on the basis of their localization and physiological roles described here. NERPs are derived from distinct regions of the neurosecretory protein VGF. In this review, we describe distribution and biological functions of NERPs, and discuss the role of VGF as a precursor of multiple bioactive peptides with diverse neuroendocrine functions.

Structures of VGF and NERPs

VGF protein is a neurosecretory protein originally identified as a product of the nerve growth factor-responsive gene *Vgf* in rat pheochromocytoma PC12 cells [14, 15]. This name is based on the selection of this clone from plate V of a nerve Growth Factor-induced PC12 cell cDNA library [14]. The *Vgf* gene is highly conserved from zebrafish to humans and encodes a 617-amino acid protein in rats, and a 615-amino acid protein in humans [15]. The human and mouse *Vgf* genes map to chromosome 7q22 and chromosome 5, respectively [16, 17]. Two introns interrupt the region encoding the 5' untranslated sequence of *Vgf*, and the entire VGF protein is encoded by exon 3 [16]. During rat development, VGF mRNA is expressed in the neural crest cells fated to become enteric ganglia at embryonic day (E)11.5 and in discrete regions of the brain and the primordia of the dorsal root, cranial, and sympathetic ganglia at E13.5 [18]. Thus, VGF expression appears to occur in the peripheral nervous system as maturing neurons cluster to form ganglia. In adult rats, VGF mRNA was detected in subsets of endocrine cells in the anterior and posterior pituitary glands, adrenal medulla, pancreas, and gastrointestinal tract [19]. VGF mRNA is also expressed in the brain, spinal cord, dorsal root ganglia, sympathetic ganglia, and enteric nervous system, being particularly abundant in the hypothalamus, especially in the preoptic, periventricular, paraventricular (PVN), supraoptic (SON), suprachiasmatic, and arcuate nuclei [19–22].

Human NERP-1 consists of 26 amino acid residues, while NERP-2 has 38 amino acid residues (Fig. 1A). The primary sequences of human and rat NERP-1 differ by four amino acids, and those of human and rat NERP-2 differ by only one amino acid. NERP-1 also exists in a short version with a single residue N-terminal deletion, while NERP-2 also exists in a six-residue N-terminal deleted form. Rat NERP-1 is 25 amino acids long and lacks the arginine residue at the N-terminus of human NERP-1. NERP-1 is derived from amino acids 281–306 of human VGF and amino acids 285–309 of rat VGF, while NERP-2 derives from amino acids 310–347 of human VGF and amino acids 313–350 of rat VGF. NERPs are novel peptides with no significant homology to any previously described peptides.

VGF protein in humans, chimpanzees, rats, and mice [14, 16, 17] have several paired, basic amino acid residues that represent potential cleavage sites for protein convertases of the kexin/subtilisin-like serine proteinase family [23]. Indeed, human and rat NERP-1 derive from processing of VGF at a typical dibasic cleavage site, ^{306}AGRR↓Q (human) or ^{309}AGRR↓Q (rat) (Fig. 1B). In contrast, human and rat NERP-2 are cleaved at a non-typical site following a single arginine within the ^{347}GGR↓G (human) or ^{350}GGR↓G (rat) sequence.

NERPs suppress vasopressin release

Radioimmunoassays (RIAs) using antibodies raised against the C-terminal regions of rat NERP-1 or NERP-2 combined with HPLC showed that both peptides are highly abundant in the rat hypothalamus [7]. The immunoreactive NERP-1 and NERP-2 contents in the whole rat brain were 6.06 ± 0.27 and 4.00 ± 0.17 fmol/mg wet weight, respectively. The whole hypothalamus contained 14.3 ± 1.1 fmol NERP-1/mg wet weight and 12.6 ± 1.5 fmol NERP-2/mg wet weight. Cell bodies with strong immunostaining of NERPs were observed in the magnocellular neurons of the SON and PVN [7], which produce the antidiuretic neuropeptide vasopressin and the reproduc-

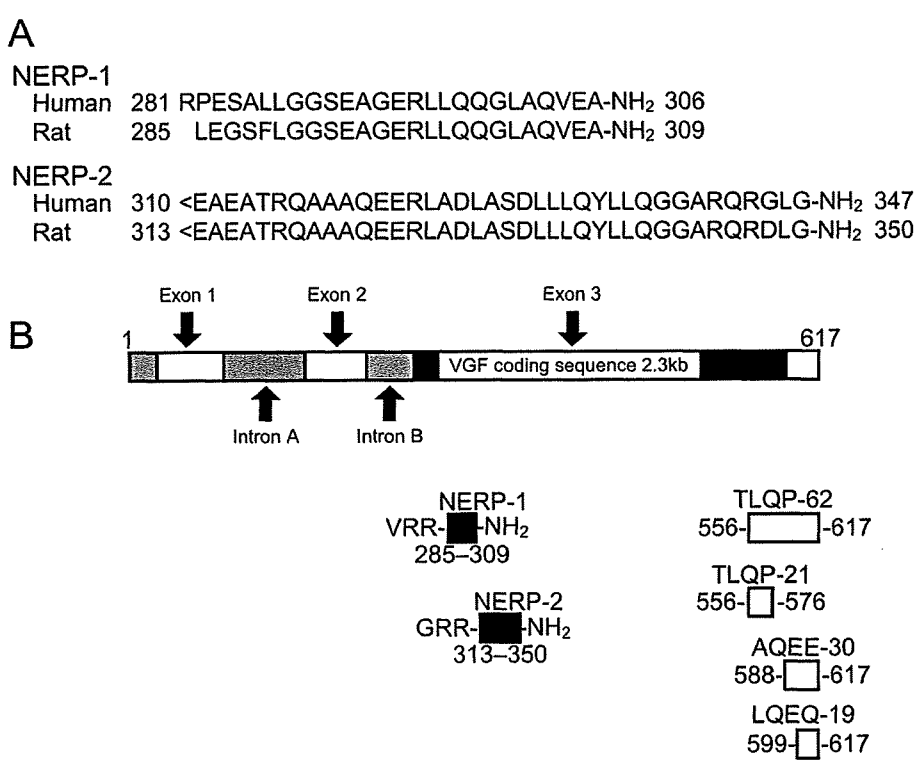

Figure 1. *(A)* Sequence alignments of human and rat NERPs. <E, pyroglutamate. *(B)* Schematic diagram of peptides derived from VGF. The numbers indicate the positions of amino acid residues in rat VGF protein. The closed boxes represent NERP-1 and NERP-2.

tive neuropeptide oxytocin [24]. Vasopressin is a 9-amino acid peptide that stimulates water reabsorption in the kidney. NERP immunoreactivity and VGF mRNA frequently colocalized with vasopressin, but rarely with oxytocin [7, 25]. Immunogold electron microscopy revealed the colocalization of NERPs with vasopressin in storage granules. In addition, immunoreactive signals for NERPs and vasopressin are colocalized in magnocellular neurons of the human SON and PVN. NERP-1 and NERP-2 both circulate in human plasma, with respective plasma concentrations of 3.5 ± 1.0 and 2.0 ± 0.4 fmol/ml. Although the vasopressin level in the plasma of normal human subjects increased during osmotic stimulus by salt-loading, the plasma levels of NERP-1 and -2 did not change.

VGF mRNA levels in both the PVN and SON were upregulated upon water deprivation in rats (PVN, 153.0 ± 13.6%; SON, 161.9 ± 12.4%; % of controls), concomitant with the upregulation of vasopressin mRNA levels. VGF mRNA levels in the PVN and SON of salt-loaded rats also increased with vasopressin mRNA level [26]. These *in vivo* and immunocytochemical observations suggest that NERPs may be involved in the central control of body fluid balance. An intracerebroventricular (icv) injection of hypertonic NaCl or angiotensin II (AII) increased plasma vasopressin levels in rats. This stimulation was suppressed in a dose-dependent manner by icv injection of NERP-1 before injection of vasopressin secretagogues. Similar but weaker effects were observed with NERP-2. Neither nonamidated NERP-1 (NERP-1-Gly) nor nonamidated NERP-2 (NERP-2-Gly) suppressed vasopressin secretion. The increase in plasma vasopressin concentrations caused by water deprivation in rats was also suppressed by icv-administered NERP-1 or NERP-2. Furthermore, immunoneutralization by icv administration of anti-NERP-1 IgG or anti-NERP-2 IgG reversed plasma vasopressin suppression induced by acute water loading, suggesting that NERPs function as endogenous peptides that regulate vasopressin secretion. Also, *in vitro* experiments using rat hypothalamic explants demonstrated that NERP-1 reversibly suppressed basal and AII-induced vasopressin secretion. NERP-2 had a similar effect, but NERP-1-Gly and NERP-2-Gly were not able to suppress vasopressin secretion. Vasopressin-producing magnocellular neurosecretory cells send their axons to the posterior pituitary, from which vasopressin is secreted into the circulatory system [24, 27]. NERPs also suppressed vasopressin secretion from the posterior pituitary in rats. Thus, NERPs may be potent endogenous suppressors of vasopressin secretion.

The neurons that produce vasopressin and oxytocin in the SON and PVN have characteristic electrophysiological properties and firing patterns [28]. Vasopressin neurons increase their firing rate both after admin-

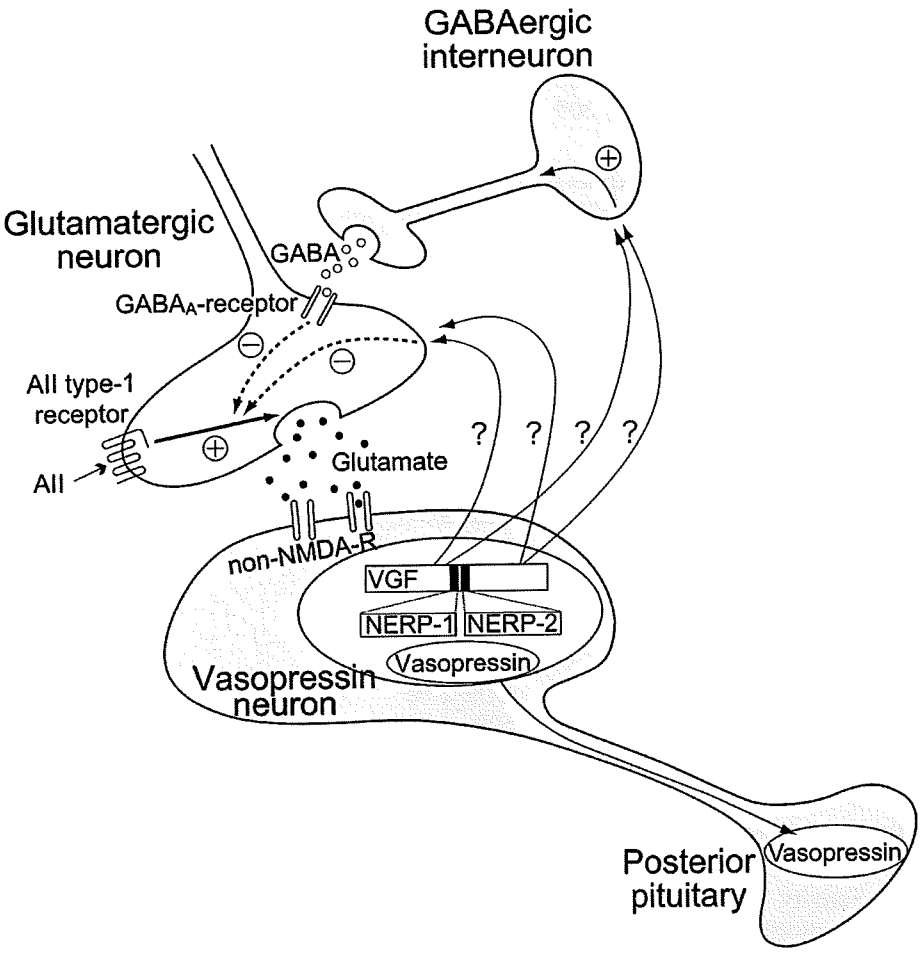

Figure 2. Proposed mechanisms by which NERPs regulate vasopressin secretion. Vasopressin is synthesized in the PVN and SON and transported to the posterior pituitary from where vasopressin is secreted into the circulation. Vasopressin secretion is regulated by excitatory glutamatergic input and inhibitory GABAergic input. AII acts on the AII type-1 receptor at the presynaptic terminal of glutamatergic neurons and increases spontaneous glutamate release, thereby activating the non-NMDA receptor. The GABA-evoked $GABA_A$ receptor-mediated response suppresses excitation of glutamatergic neurons. NERPs localize in the vasopressin neurons and suppress AII-induced vasopressin release. NERPs may suppress presynaptic release of glutamate or stimulate GABA release. **non-NMDA-R**, non-N-methyl-D-aspartate receptor.

istration of hyperosmotic saline and non-osmotic stimuli such as hemorrhaging [29, 30]. The major neural signals that regulate vasopressin neurons are presynaptic release of glutamate and γ-aminobutyric acid (GABA). Whole-cell patch clamp recording of the excitatory postsynaptic currents in the SON suggests that AII increases presynaptic glutamate release [31]. Hypertonic saline also stimulates vasopressin release through activation of non-N-methyl-D-aspartate (NMDA) receptor expressed on the anteroventral third ventricular region [32]. Although cell-surface receptors and target proteins of NERPs have not yet been identified, the action of NERPs in suppression of AII- and NaCl-induced vasopressin release suggests that they presynaptically inhibit glutamatergic inputs or enhance GABAergic inputs to vasopressin neurons (Fig. 2). Further investigation using whole-cell patch clamp recordings of PVN or SON slice preparations to examine the effect of NERPs on synaptic inputs to vasopressin neurons should elucidate the mechanisms by which NERPs modulate vasopressin release.

Phenotype of *Vgf*$^{-/-}$ mice and the activities of other VGF-derived peptides

Vgf$^{-/-}$ mice showed a significant lack of body weight gain at postnatal day 3 [16]. After weaning, the body weight of *Vgf*$^{-/-}$ mice was 50–70% that of wild-type littermates. Whereas *Vgf*$^{-/-}$ mice showed increased oxygen consumption compared to wild-type and *Vgf*$^{+/-}$ littermates, they had normal core body temperature and sympathetic tone and low circulating thyroid hormone levels [16]. Energy homeostasis is regulated by distinct hypothalamic regions: i) the arcuate nucleus, which contains populations of neurons expressing the orexigenic factors neuropeptide Y (NPY) and agouti-related protein (AgRP) [33, 34], and the anorexigenic factors pro-opiomelanocortin (POMC) (from which α-melanocyte stimulating hormone derives) and cocaine-amphetamine regulated transcript [35]; ii) the lateral hypothalamus, which contains the orexigenic peptides orexin-A and -B [2] (also known as hypocretin-1 and -2 [36]) and melanin-concentrating hormone [37]; and iii) the PVN, which contains the anorexigenic corticotropin-releasing hormone [38]. The mRNA level of