There was no typical GC or CAAT box but there were a TATATAA element (−585 to −579) and putative binding sites for several transcription factors including activator protein-2 (AP2) (−1897 to −1890, −1851 to −1844, −1738 to −1731, −1393 to −1384, −1308 to −1301, −1179 to −1172, −1157 to −1152, −744 to −735, −648 to −641, −371 to −365, and −264 to −254), basic helix-loop-helix (bHLH) (−1837 to −1832, −1539 to −1534, −973 to −968, −768 to −763, −759 to −753, −236 to −231, −132 to −127, and −110 to −105), PEA-3 (−1516 to −1511), Myb (−962 to −957), NF-IL6 (−1636 to −1628), half-site for the estrogen response element (−876 to −871, −715 to −710), half-site for the glucocorticoid response element (−682 to −677), hepatocyte nuclear factor-5 (−1361 to −1354), and NF-κB (−1057 to −1048).

Functional analysis of the human ghrelin 5′-flanking region

Plasmids containing cloned 5′-flanking sequences of the human ghrelin gene fused to the luciferase gene were transfected into several cell lines. As illustrated in Fig. 4A, the human ghrelin promoter is activated only in ECC10 derived from human gastric carcinoid [19], but not activated in other examined cell lines including MKN1 and MKN45 which were derived from human gastric adenocarcinoma, suggesting that human ghrelin promoter has cell-specific activity.

To identify the important regulatory regions for the expression of human ghrelin gene, deletion mutants of the 5′-flanking sequences were constructed. Fig. 4B summarizes the effect of these deletions on the luciferase reporter activity in ECC10. The luciferase activity was increased by deletion from −2000 to −605, whereas it was decreased by further deletion. Because there was a TATATAA element from −585 to −579, we mutated or deleted this element to clarify its role for the activation of human ghrelin promoter. Neither mutation nor deletion of the TATATAA element decreased the promoter activity, suggesting that TATATAA element is not functioning (Fig. 4C).

Fasting causes the increase of both ghrelin and glucagon levels in peripheral circulation. Therefore, we examined the effect of glucagon and its second messen-

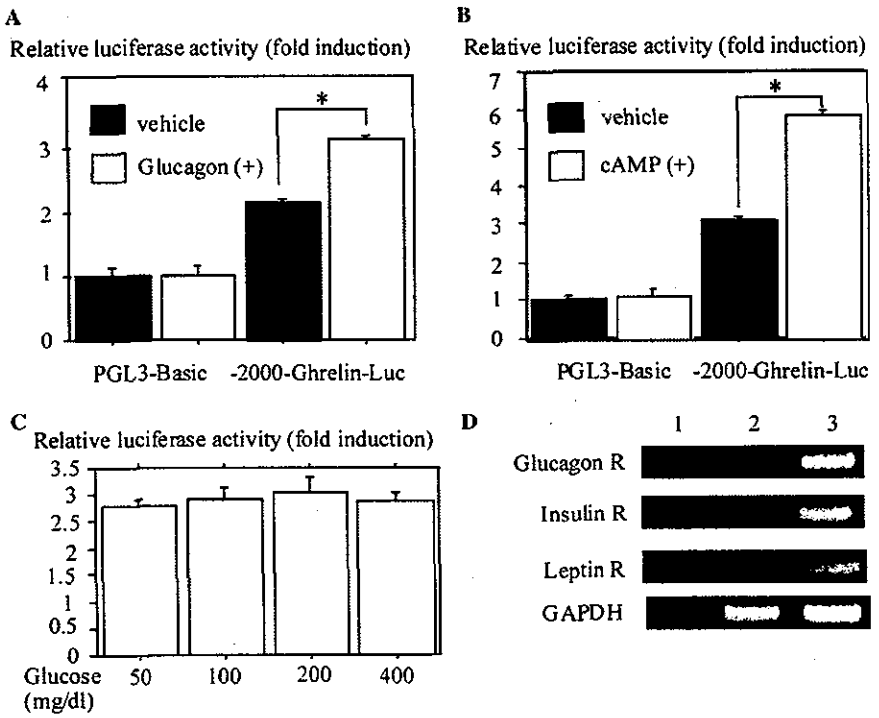

Fig. 5. (A) Effect of glucagon on the reporter activity of −2000-Ghrelin-Luc containing from −2000 to −1 of the human ghrelin gene. PGL3-Basic and −2000- Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 0.1% BSA with or without 10 nM glucagon. (B) Effect of CPT-cAMP on the reporter activity of −2000-Ghrelin-Luc. PGL3-Basic and −2000-Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 10% FCS with or without 1 mM CPT-cAMP. (C) Reporter activity of −2000-Ghrelin-Luc cultured in several different glucose concentrations. PGL3-Basic and −2000-Ghrelin-Luc were transfected into ECC10 and cultured in RPMI 1640 containing 10% FCS with glucose (50, 100, 200, or 400 mg/dl). (A–C) In each transfection, 2 μg of the reporter plasmids was transfected into the ECC10 cells cultured in the 35-mm dishes. Twenty nanograms of pRL-CMV containing the cDNA encoding *Renilla* luciferase was co-transfected to normalize the luciferase activity in each transfection. Each bar represents the means ± SE ($n = 5$) of luciferase activity expressed as fold induction vs. promoterless PGL3-Basic. Asterisks mean statistical significance vs. vehicle-treated control ($P < 0.05$). (D) Expression of mRNA of glucagon receptor, insulin receptor, leptin receptor, and G3PDH in ECC10 (lane 2) and in human normal liver (lane 3). Autoclaved water as a negative control was used (lane 1).

ger cAMP on the human ghrelin promoter activity. Glucagon and cAMP significantly increased the activity of human ghrelin promoter (Figs. 5A and B). In contrast, the concentration of glucose in the medium did not affect the promoter activity (Fig. 5C). ECC10 expressed the glucagon receptor but not the receptor of insulin nor leptin (Fig. 5D), suggesting that insulin and leptin seem not able to regulate ghrelin gene transcription directly, at least in our experimental conditions.

Discussion

This is the first report of the functional analysis of 5′-flanking region of the human ghrelin gene. Although Tanaka et al. [20] reported the presence of short noncoding first exon of mouse ghrelin gene and suggested a possible function of TATATAA element detected in mouse ghrelin promoter, we and another group [1] could not detect such a noncoding exon in the human ghrelin gene. Furthermore, here we showed that TATATAA element detected in the 5′-flanking region of the human ghrelin gene is not functioning at least under our experimental conditions. Since Tanaka et al. did not perform a functional analysis of the mouse ghrelin promoter, it still remains unclear whether TATATAA element is working in mouse ghrelin gene.

Although ECC10 is derived from human gastric carcinoid [19] but not from X/A cells which produce ghrelin in the normal stomach [5], RT-PCR showed ghrelin mRNA in ECC10 while not in MKN1 and MKN45 which are derived from human gastric adenocarcinoma (data not shown). Furthermore, among several cell lines examined, ghrelin promoter is activated only in ECC10 and not activated in other cell lines including MKN1 and MKN45, suggesting that human ghrelin promoter has cell-specific activity.

The plasma ghrelin levels were increased during fasting and decreased by refeeding. However, the factors regulating ghrelin production have not been clarified. We first proposed the possibility that glucagon is a positive regulator of ghrelin production. We also showed that cAMP-signaling up-regulates the ghrelin gene transcription. It is noteworthy that GHRH, which also activates a cAMP-signaling cascade, up-regulates ghrelin gene transcription in the pituitary gland [21]. However, the mechanism by which cAMP up-regulates ghrelin gene transcription remains unclear because there are no AP1 site and cAMP responsive element (CRE) in our cloned 5′-flanking region at least from −2000 to −1 upstream from the translation start site. It is possible that there is a cell-specific transcription factor that regulates ghrelin gene transcription and integrates cAMP stimulation to activate ghrelin gene transcription using transcriptional cofactors in the same manner as other cell specific transcription factors [14,22,23].

In addition to stimulating the secretion of GH, ghrelin also regulates the energy balance [1–3]. Therefore, several factors involved in the energy balance may regulate the ghrelin production. Ghrelin expression in the stomach is reported to be increased by insulin and leptin [11]. Later, there were also reports of contradictory results that gastric ghrelin expression was decreased by insulin [12] and leptin [13]. Therefore, insulin and leptin do not seem to be the main regulators for the production of ghrelin. Furthermore, in ECC10 cells neither leptin nor insulin seemed to regulate the ghrelin gene transcription at least directly through their receptors.

In summary, we have characterized the 5′-flanking region of the human ghrelin gene for the first time. These initial characterizations should facilitate further study of the mechanism involved in the transcriptional regulation of the human ghrelin gene expression in human health and disease.

Acknowledgments

We thank Dr. Motoyama for his permission for Riken Cell Bank to distribute ECC10 cells and Miss Chika Ogata for excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from Japanese Ministry of Education, Science, Sports and Culture, and grants from Japanese Ministry of Health, Labour and Welfare and from Growth Science Research Foundation.

References

[1] 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.
[2] M. Tschop, D.L. Smiley, M.L. Heiman, Ghrelin induces adiposity in rodents, Nature 407 (2000) 908–913.
[3] M. Nakazato, N. Murakami, Y. Date, M. Kojima, H. Mastuo, K. Kangawa, S. Matsukura, A role for ghrelin in the central regulation of feeding, Nature 409 (2001) 194–198.
[4] H. Ariyasu, K. Takaya, T. Tagami, Y. Ogawa, K. Hosoda, T. Akamizu, M. Suda, T. Koh, K. Natsui, S. Toyooka, G. Shirakami, T. Usui, A. Shimazu, K. Doi, H. Hosoda, M. Kojima, K. Kangawa, K. Nakao, Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans, J. Clin. Endocrinol. Metab. 86 (2001) 4753–4758.
[5] Y. Date, M. Kojima, H. Hosoda, A. Sawaguchi, M.S. Mondal, T. Suganuma, S. Matsukura, K. Kangawa, M. Nakazato, Ghrelin, a novel growth-hormone-releasing acylated peptide, is a distinct endocrine cell type in the gastro-intestinal tracts of rats and humans, Endocrinology 141 (2000) 4255–4261.
[6] K. Mori, A. Yoshimoto, K. Takaya, K. Hosoda, H. Ariyasu, K. Yahata, M. Mukoyama, A. Sugawara, H. Hosoda, M. Kojima, K. Kangawa, K. Nakao, Kidney produces a novel acylated peptide, ghrelin, FEBS Lett. 486 (2000) 213–216.
[7] O. Gualillo, J. Caminos, M. Blanco, T. Garcia-Caballero, M. Kojima, K. Kangawa, C. Dieguez, F. Casanueva, Ghrelin, a novel placental-derived hormone, Endocrinology 142 (2001) 788–794.
[8] Y. Date, M. Nakazato, S. Hashiguchi, K. Dezaki, M.S. Mondal, H. Hosoda, M. Kojima, K. Kangawa, T. Arima, H. Matsuo, T.

Yada, S. Matsukura, Ghrelin is present in pancreatic α-cells of humans and rats and stimulates insulin secretion, Diabetes 51 (2002) 124–129.
[9] M. Korbonits, S.A. Bustin, M. Kojima, S. Jordan, E.F. Adams, D.G. Lowe, K. Kangawa, A.B. Grossmann, The expression of the growth hormone receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors, J. Clin. Endocrinol. Metab. 86 (2001) 881–887.
[10] M. Papotti, P. Cassoni, M. Volante, R. Deghenghi, G. Muccioli, E. Ghigo, Ghrelin-producing endocrine tumors of the stomach and intestine, J. Clin. Endocrinol. Metab. 86 (2001) 5052–5059.
[11] K. Toshinai, M.S. Mondal, M. Nakazato, Y. Date, N. Murakami, M. Kojima, K. Kangawa, S. Mastukura, Upregulation of ghrelin expression in the stomach upon fastion, insulin-induced hypoglycemia, and leptin administration, Biochem. Biophys. Res. Commun. 281 (2001) 1220–1225.
[12] K.C. McCowen, J.A. Maykel, B.R. Bristrian, P.R. Ling, Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents, J. Endocrinol. 175 (2002) R7–R11.
[13] A. Asakawa, A. Inui, T. Kaga, H. Yuzuriha, T. Nagata, N. Ueno, S. Makino, M. Fujimiya, A. Niijima, M.A. Fujino, M. Kasuga, Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin, Gastroenterology 120 (2001) 337–345.
[14] M. Kishimoto, Y. Okinura, K. Yagita, G. Iguchi, M. Fumoto, K. Iida, H. Kaji, H. Okamura, K. Chihara, Novel function of the transactivation domain of a pituitary-specific transcription factor, Pit-1, J. Biol. Chem. 47 (2002) 45141–45148.
[15] G. Albertin, F. Aragona, L. Gottardo, L.K. Malendowicz, G.G. Nussdorfer, Human pheochromocytomas, but not adrenal medulla, express glucagon-receptor gene and possess an in vitro secretory response to glucagon, Peptides 22 (2001) 597–600.
[16] M. Takahashi, T. Yamada, I. Tooyama, I. Moroo, H. Kimura, T. Yamamoto, H. Okada, Insulin receptor mRNA in the substantia nigra in Parkinson's disease, Neuroscience 204 (1996) 201–204.
[17] A. Glasow, A. Haidan, U. Hilbers, M. Breidert, J. Gillespie, W.A. Scherbaum, G.P. Chrousos, S.R. Bornstein, Expression of ob receptor in normal human adrenals: differential regulation of adrenocortical and adrenomedullary function by leptin, J. Clin. Endocrinol. Metab. 83 (1998) 4459–4466.
[18] J.Y. Tso, X.H. Sun, T.H. Kao, K.S. Reece, R. Wu, Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene, Nucleic Acids Res. 13 (1985) 2485–2502.
[19] T. Fujiwara, T. Motoyama, N. Ishihara, H. Watanabe, T. Kumanishi, K. Kato, H. Ichinose, T. Nagatsu, Characterization of four new cell lines derived from small-cell gastrointestinal carcinoma, Int. J. Cancer 54 (1993) 965–971.
[20] M. Tanaka, Y. Hayashida, T. Iguchi, N. Nakao, N. Nakai, K. Nakashima, Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon, Endocrinology 142 (2001) 3697–3700.
[21] J. Kamegai, H. Tamura, T. Shimizu, S. Ishii, H. Sugihara, S. Okikawa, Regulation of the ghrelin gene: growth hormone-releasing hormone upregulates ghrelin mRNA in the pituitary, Endocrinology 142 (2001) 4154–4157.
[22] K. Zanger, L.E. Cohen, K. Hashimoto, S. Radovick, F.E. Wondisford, A novel mechanism for cyclicadenosine 3′5′-monophosphate regulation of gene expression by CREB-binding protein, Mol. Endocrinol. 13 (1999) 268–275.
[23] K. Hashimoto, K. Zanger, A.N. Hollenberg, L.E. Cohen, S. Radovick, F.E. Wondisford, cAMP response element-binding protein mediates thyrotropin-releasing hormone signaling on thyrotropin subunit genes, J. Biol. Chem. 275 (2000) 33365–33372.

European Journal of Endocrinology (2003) 148 333–338 ISSN 0804-4643

CLINICAL STUDY

A study of carotid intima-media thickness in GH-deficient Japanese adults during onset among adults and children

Masahiro Murata, Hidesuke Kaji[2], Ishikazu Mizuno, Tatsuya Sakurai, Keiji Iida, Yasuhiko Okimura[1] and Kazuo Chihara

Third Division, Department of Medicine, and [1] Department of Basic Allied Medicine, Kobe University School of Medicine, Kobe, 650-0017, Japan and [2] Department of Physiology/Metabolism, College of Nursing Art and Science, Hyogo, Akashi 673-8588, Japan

(Correspondence should be addressed to H Kaji; Email: hidesuke_kaji@cnas-hyogo.ac.jp)

Abstract

Objectives: Increased carotid intima-media thickness (IMT) has been reported among Caucasian adult GH-deficient (AGHD) patients, but not Japanese. Also, it is known that the clinical and biochemical characteristics of AGHD patients are somewhat different based on the onset of the disease in either childhood or adult life. Nevertheless, there has been no study comparing the magnitude of the deviation of their IMT from normal subjects between child-onset (CO) and adult-onset (AO) patients in terms of Z score. The aim of this study, therefore, was first to examine whether Japanese AGHD patients have a risk of early development of atherosclerosis similar to Caucasian patients and secondly to assess the difference in the onset and in progression of atherosclerosis.

Design and subjects: Thirty-four patients (17 CO-AGHD, age 29±7 years, body mass index (BMI) 24±3.8 kg/m^2 and 17 AO-AGHD, age 48±12 years, BMI 23±3.6 kg/m^2) and 34 age- and sex-matched healthy controls (17 CO controls and 17 AO controls) were enrolled in the present study. Blood samples were taken for measurements of lipids, lipoproteins and IGF-I. Subsequently, patients underwent IMT assessment.

Results: CO patients were significantly younger than AO patients. The duration of GH-deficiency in CO patients was significantly longer than that in AO patients. Serum triglyceride (TG) was significantly higher in CO patients than in CO controls ($P < 0.05$). Serum total cholesterol and TG were significantly higher in AO patients than in AO controls ($P < 0.01$). The IMT was significantly greater in CO and AO patients (0.82±0.08 and 0.79±0.03 mm) than in CO and AO controls (0.59±0.02 and 0.68±0.03 mm, $P < 0.01$ and $P < 0.01$ respectively). There was no significant difference in raw values of IMT between CO and AO patients. However, the Z score of IMT calculated using normal Japanese IMT values was significantly higher in CO than in AO patients (2.07±0.68 vs 0.35±0.48, $P < 0.05$).

Conclusions: These findings suggest that GH deficiency appears to increase an atherosclerotic risk in Japanese AGHD patients, as with Caucasians, and to cause more extensive IMT thickening in CO-AGHD than AO-AGHD patients.

European Journal of Endocrinology **148** 333–338

Introduction

Recent retrospective studies have revealed that adult patients with hypopituitarism under conventional hormone replacement treatment by thyroid hormone, adrenal and/or sex steroids show a higher mortality rate, mostly attributable to vascular disorders, and higher morbidity from diseases related to atherosclerosis than general healthy subjects (1). The early development of atherosclerosis in these patients is thought to be at least partly caused by growth hormone (GH) deficiency. The severity of atherosclerosis has been assessed by measurement of carotid intima-media thickness (IMT), a well-accepted marker of vascular risks, by means of high resolution ultrasonography. IMT was found to increase in adult Caucasian patients with hypopituitarism (2) as well as child-onset (CO) adult GH-deficient (AGHD) patients (3). Furthermore, a 1 year GH treatment of AGHD patients resulted in a significant amelioration of their increased IMT (4).

GH-deficient (GHD) patients also have associated abnormal metabolism of lipid and carbohydrate, which may contribute to an increased risk of vascular disease (5–13). Administration of GH to these patients reduced adipose tissue and increased lean body mass (14, 15), increased physical and cardiac performance (16–19), normalized lipid metabolism (9) and improved quality of life (20, 21). However, AGHD may not be a

homogeneous clinical entity because clinical characteristics of AGHD appear to be somewhat different between CO and adult-onset (AO) patients. Attanasio *et al.* (22) reported that the body height, weight, body mass index (BMI), lean body mass and the waist/hip ratio of AO patients were all significantly greater than in CO patients but there were no differences in serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels between the two groups. We wondered whether the atherosclerotic risk is similar among AO and CO patients. To the best of our knowledge, there is no report comparing the Z score of IMT among AO and CO patients so far. Furthermore, IMT in Japanese AGHD patients remains not definitively studied.

The aim of this study was to assess the progression of atherosclerosis by means of IMT measurement in Japanese AGHD patients and to analyse how it differs in a comparison between Japanese CO and AO patients.

Patients and methods

Patients

Thirty-four patients (17 CO, 10 males and 7 females, age 29±7 years, height 1.62±0.02 m, BMI 24±3.8 kg/m^2 and 17 AO, 10 males and 7 females, age 48±12 years, height 1.61±0.02 m, BMI 23±3.6 kg/m^2) and 34 healthy controls (17 CO controls and 17 AO controls), comparable in gender and age distribution were enrolled in the present study (Table 1). The aetiology of hypopituitarism was varied among the patients as shown in Table 1. In all patients, GH deficiency was diagnosed as peak serum GH levels below 3 μg/l in insulin tolerance tests.

All CO patients were treated with GH at the dose of 0.5 IU/kg body weight per week for a period ranging from 1 to 11 years, but GH administration discontinued at least 3 years before entry into the study, whereas none of the AO patients had been given GH therapy (Table 1). All patients had multiple pituitary deficiency and were under replacement therapy with various hormones such as thyroxine, hydrocortisone or desmopressin at standard doses. All patients with hypogonadism below age 50 except four CO and two AO patients had sex hormone replacement. No medications other than hormones were prescribed. Patients known to have diabetes, cardiovascular diseases or hypertension were excluded. All participants gave their informed consent, and the protocol was approved by the local institutional review board in our hospital.

Protocol

All participants were studied in the postabsorptive state after a 12 h overnight fast. Blood pressure (systolic and

Table 1 Characteristics of the study population. Values are means±S.D.

	CO controls	CO-AGHD	AO controls	AO-AGHD
No.	17	17	17	17
Age (years)	29±7	29±7[b]	48±12	48±12
Sex (M/F)	10/7	10/7	10/7	10/7
Height (m)	1.67±0.07	1.62±0.08[a]	1.64±0.02	1.61±0.02[c]
BMI (kg/m^2)	22±1.6	24±3.8[a]	22±3.2	23±3.6
Smoking	2/17	2/17	2/17	2/17
Duration of GHD	–	21±9[b]	–	14±9
Duration of GH treatment	–	7±4	–	0
PAS (mmHg)	109±15	108±15	115±7	111±11
PAD (mmHg)	62±9	71±11	73±9	72±12
Aetiology (no.)				
Pituitary adenoma	–	1	–	12
Craniopharyngioma	–	3	–	1
Germinoma	–	5	–	0
Optic glioma	–	1	–	0
Sheehan's syndrome	–	0	–	1
Trauma	–	1	–	2
Idiopathic	–	6	–	1
Replacement treatment				
Thyroxine	–	15	–	14
Cortisol	–	14	–	14
Sex steroids	–	9	–	7
Desmopressin	–	6	–	1
TC (mg/dl)	188±15	211±48	186±16	209±24[c]
LDL-C (mg/dl)	109±14	126±35	111±14	119±19
HDL-C (mg/dl)	60±9	55±18	59±10	61±24
Total TG (mg/dl)	58.8±27.6	159±121[a]	78.3±36.2	143±79[c]
IGF-I (ng/ml)	–	63±35	–	60±24
Fasting glucose (mg/dl)	81±12	80±13	82±9	80±10

[a]$P < 0.05$ vs CO control, [b]$P < 0.01$ vs AO-AGHD, [c]$P < 0.01$ vs AO control. PAS, systolic blood pressure; PAD, diastolic blood pressure.

diastolic) was measured using the sphygmomanometric method. Blood samples were taken for measurements of lipids, lipoproteins and insulin-like growth factor-I (IGF-I). Then, patients underwent ultrasonographic scanning of the carotid arteries according to a recent Japanese study (24) by a trained physician and were photographed. The assessment of IMT was performed by a high resolution echo-colour Doppler system (LOGIQ700MR; GE Yokogawa Medical System, Tokyo, Japan). Scanning of the extracranial carotid arteries in the neck was performed bilaterally in three different longitudinal projections: anterior-oblique, lateral and posterior-oblique as well as the transverse projection. Three determinations of intima plus medial thickness were conducted at the site of the greatest thickness and at two points, 1 cm upstream and 1 cm downstream from the site of the greatest thickness. These three values were averaged. The greatest value among the six averaged intima plus medial thickness (three from the left and three from the right) was used as the representative IMT value for each individual. The coefficients of variation of the measurements were less than 3%. All scans were read by an independent physician, blinded as to the clinical status of the subjects. This method is well-known and valid in Japan, although performed in a different way from most previous investigation of IMT in GHD patients. The Z score of IMT was defined as the standard deviation of mean IMT values in normal Japanese of every 10 year age cohort (23).

Serum TC, HDL-C, serum triglyceride (TG) and plasma glucose levels were assayed by standard laboratory techniques. LDL-C was estimated by the Friedewald equation. Serum IGF-I levels were determined by an IRMA after extraction (SRL, Inc., Tokyo, Japan).

Statistical analysis

Data are expressed as means±S.E.M. in Figures and means±S.D. in the Table. Statistical analysis was performed using one-way repeated measures ANOVA in both the Table and Fig. 1, and using Student's *t*-test in Fig. 2. $P < 0.05$ was considered significant.

Results

The comparison of the background data for the CO and AO patients revealed the age and the duration of GH deficiency to be significantly different among the two groups. The average age of CO patients was significantly younger than that of AO patients ($P < 0.001$), and the duration of GH deficiency in CO patients was significantly longer than in AO patients ($P < 0.01$). However, all CO patients had a past history of GH treatment to ameliorate their short stature. The duration of GH administration was varied among patients. The spectrum of causes of GH deficiency was entirely different between CO and AO patients. The prevalence of cigarette smoking, blood pressure and hormone replacement were similar among the CO and AO patients (Table 1).

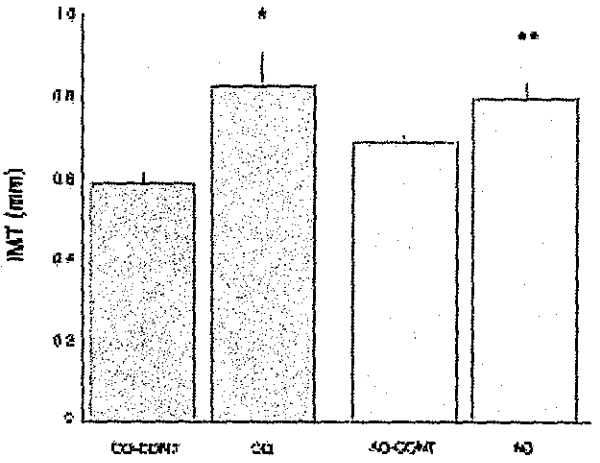

Figure 1 Carotid IMT in CO-AGHD, CO control, AO-AGHD and AO control. The values represent the means±S.E.M. *$P < 0.01$ vs CO control; **$P < 0.01$ vs AO control.

Furthermore, compared with the data of control subjects, height was significantly shorter in CO patients than in CO controls and was significantly shorter in AO patients than in AO controls ($P < 0.05$). BMI was significantly higher in CO patients than CO controls ($P < 0.01$), but not significantly different among AO patients and AO controls. Serum TC, LDL-C, HDL-C and glucose were similar in CO patients and CO controls. Serum TG was significantly higher in CO patients than in CO controls ($P < 0.05$). Serum LDL-C, HDL-C and glucose were similar in AO patients and AO controls. Serum TC and TG were significantly higher in AO patients than in AO controls ($P < 0.01$ and $P < 0.01$ respectively). However, no difference was observed in serum TC, LDL-C, HDL-C, TG, IGF-I and glucose and in BMI among CO and AO patients (Table 1).

The IMT in AGHD patients was significantly greater than that in control subjects irrespective of the onset of their GH deficiency (CO patients, 0.82±0.08 mm vs CO controls, 0.59±0.02 mm, $P < 0.01$; AO patients, 0.79±0.03 mm vs AO controls, 0.68±0.03 mm, $P < 0.01$) (Fig. 1). Height and BMI are major determinants of IMT. CO and AO patients were significantly shorter in height in comparison with the respective controls (Table 1). Therefore, IMT was corrected by height. IMT/height was still significantly greater in CO patients than in CO controls (0.508±0.20 vs 0.365±0.064, $P < 0.01$). IMT/height was also significantly greater in AO patients than in AO controls (0.491±0.0.028 vs 0.425±0.021, $P < 0.05$). Since BMI was significantly higher in CO patients than CO controls, IMT was corrected by BMI in CO patients

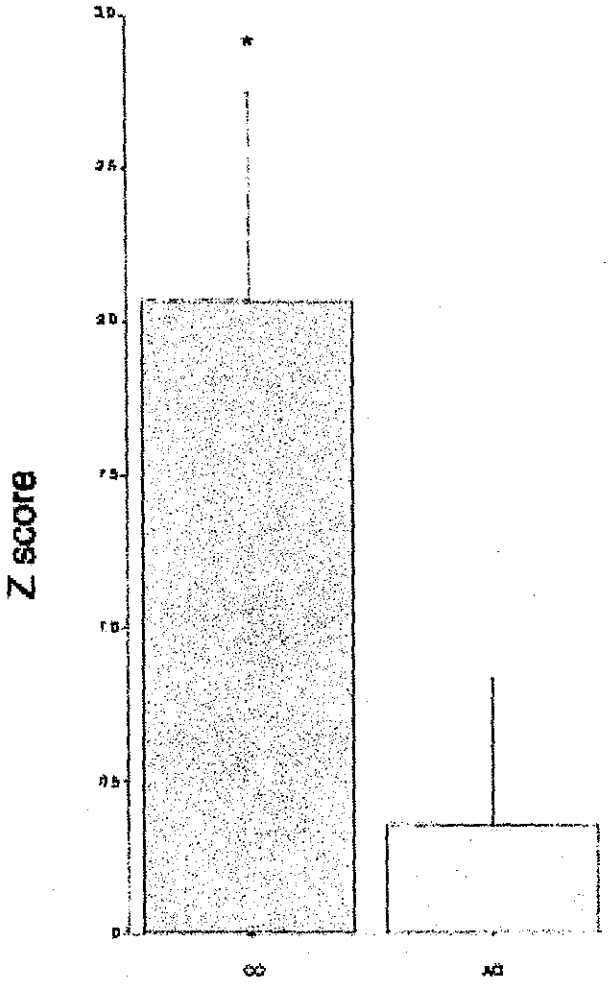

Figure 2 Comparison of the Z score of IMT in CO-AGHD and AO-AGHD. The values represent the means±S.E.M. *$P < 0.05$ vs AO-AGHD.

and CO controls. IMT/BMI was still significantly higher in CO patients than CO controls (0.036±0.016 vs 0.029±0.007, $P < 0.05$). There was no significant difference in absolute values of IMT among CO and AO patients. However, the IMT increases as a variable dependent on age in normal subjects, so the Z score of the IMT was calculated in each patient. The Z score of IMT was significantly higher in CO patients than in AO patients (2.07±0.68 vs 0.35±0.48, $P < 0.05$) (Fig. 2).

Discussion

It is well known that GH deficiency causes increased body fat with a decrease in lean body mass and abnormal levels of serum lipids and lipoproteins irrespective of racial difference (24). In addition, increased insulin resistance in peripheral tissues, decreased fibrinolytic activities, abnormal cardiac structure and performance, and premature atherosclerosis with increased arterial IMT have been reported in Caucasian patients with GH deficiency (1–3, 7, 13, 16), all of which might be responsible for an increased incidence of cardiovascular morbidity and mortality (1).

Asian people, including Japanese, have their own customs and conventions, different from Caucasians', particularly preference for food, and furthermore, the body constitution of Asian people is also different from that of Caucasian people, probably due to differences in genetic and environmental backgrounds. Therefore, it is of considerable interest whether Asian patients with GH deficiency are exposed to the risk of premature atherosclerosis in the same way as Caucasian patients. There are no convincing reports regarding the morbidity and mortality in Asian patients with GH deficiency.

To assess the development of premature atherosclerosis, we decided to measure the IMT of common carotid arteries, since an increased IMT is known to be the most sensitive parameter of atherosclerotic changes and to be detected without obvious abnormalities of the classic vascular risk factors (23, 25–27). In this study, we found that the IMT of the common carotid arteries was significantly increased in AGHD irrespective of timing of the disease onset. Our findings were not completely consistent with the findings of the pioneer study in Caucasian patients by Markussis *et al.* (2). The IMT in our AO-AGHD patients was 0.79 mm on the average, which was comparable to 0.72 mm, the value in their patients aged 40–60 years. In contrast, the IMT, 0.82 mm on the average, in our CO-AGHD patients aged 29±7 years seems to be unexpectedly great since the IMT was 0.50 mm among the patients less than 40 years of age in Markussis' study. Besides, our IMT data of CO-AGHD patients were consistent with a more recent report by Capaldo *et al.* (3) that the IMT was as great as 0.83 mm in the CO-AGHD aged 25±1 years, comparable to that in our patients. These discrepancies may simply be caused by the difference among methods for IMT measurement. Our method is, of course, a valid (23) and previously reported method.

On the other hand, there is no report comparing the IMT of AGHD patients of CO and AO in one study. As mentioned above, our CO- and AO-AGHD patients showed greater IMT of the carotid arteries than the respective control subjects. There was no significant difference in the absolute values of IMT between CO and AO patients. This finding appeared to be unusual since the carotid artery IMT would be age-dependently increased in normal subjects. Then we further calculated the Z score of the IMT in each AGHD patients using the normal Japanese IMT values of every 10 year age cohort (23). It is of interest that the Z score of IMT was significantly higher in CO patients than

AO patients, suggesting that CO patients have a greater risk of early development of atherosclerosis.

The reason why premature atherosclerosis is more prevalent in CO patients continues to be unclear. The simple explanation would be that these findings are due to the longer duration of GH deficiency. Indeed, the duration of GH deficiency in our CO patients was significantly longer than that in AO patients.

Borson-Chazot *et al.* (4) reported that a 1-year GH treatment resulted in significant reduction in increased IMT of carotid arteries in GHD patients, giving strong evidence that GH itself affects carotid IMT. Their findings also indicate that carotid IMT is not completely fixed but rather reversible and changeable under certain circumstances. Carotid IMT is affected by many factors including serum lipids. In our study, serum lipid levels in CO patients tended to be higher than those in AO patients, although the difference was statistically not significant (Table 1). Furthermore, it is well known that nitric oxide (NO) is a mediator of vasodilatation, inhibition of platelet aggregation, leukocyte adhesion and inhibition of vascular smooth muscle cell growth. IGF-I induces NO in vascular endothelial cells and NO may mediate haemodynamic effects of recombinant GH in GHD patients (28, 29). In this study, IGF-I was really low in AGHD although we did not measure IGF-I levels in normal subjects who were of normal height. We thought that GHD patients indeed would have lower plasma IGF-I levels as well as NO production than normal subjects. Although IGF-I levels did not differ between CO and AO patients (Table 1), CO patients were significantly younger than AO patients. Since it is well known that IGF-I levels progressively decline with increasing age, it would be biologically plausible that IGF-I levels in CO patients were markedly lower than in AO patients. Indeed, this difference could explain why CO patients had a higher Z score of IMT than AO patients. Hence, an enhanced effect through the combination of GHD as well as lower IGF-I levels for age and lipid abnormalities may simply be attributed to the greater Z score of IMT in CO patients.

In summary, this study demonstrated for the first time that carotid IMT in Japanese AGHD patients is increased as much as in Caucasian patients. Furthermore, Japanese CO-AGHD patients showed greater IMT than AO-AGHD patients, although the reason remains to be clarified.

Acknowledgements

We are grateful to Miss Chika Ogata for excellent technical assistance. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Health and Welfare, and Growth Science Foundation in Japan.

References

1 Rosen T & Bengtsson BA. Premature mortality due to cardiovascular disease in hypopituitarism. *Lancet* 1990 **336** 285–288.
2 Markussis V, Beshyah SA, Fisher C, Sharp P, Nicolaides AN & Johnston DG. Detection of premature atherosclerosis by high-resolution ultrasonography in symptom-free hypopituitary adults. *Lancet* 1992 **340** 1188–1192.
3 Capaldo B, Patti L, Oliviero U, Longobardi S, Pardo F, Vitale F *et al.* Increased arterial intima-media thickness in childhood-onset growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 1378–1381.
4 Borson-Chazot F, Serusclat A, Kalfallah Y, Ducottet X, Sassolas G, Bernard S *et al.* Decrease in carotid intima-media thickness after one year growth hormone (GH) treatment in adults with GH deficiency. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 1329–1333.
5 Merimee TJ, Hollander W & Fineberg SE. Studies of hyperlipidemia in the HGH-deficient state. *Metabolism: Clinical and Experimental* 1972 **21** 1053–1061.
6 Libber SM, Plotnik LP, Johanson AJ, Blizzard RM, Kwiterovich PO & Migeon CJ. Long-term follow-up of hypopituitary patients treated with human growth hormone. *Medicine* 1990 **69** 46–55.
7 Rosen T, Eden S, Larson G, Wilhelmsen L & Bengtsson B-Å. Cardiovascular risk factors in adult patients with growth hormone deficiency. *Acta Endocrinologica* 1993 **129** 195–200.
8 DeBoer H, Blok GJ, Voerman HJ, Philips M & Schouten JA. Serum lipid levels in growth hormone-deficient men. *Metabolism: Clinical and Experimental* 1994 **43** 199–202.
9 Cuneo RC, Salomon F, Watts GF, Hesp R & Sönksen PH. Growth hormone treatment improves serum lipids and lipoproteins in adults with growth hormone deficiency. *Metabolism: Clinical and Experimental* 1993 **42** 1519–1523.
10 Snel YEM, Doerga ME, Brummer RJM, Zelissen PMJ & Koppeschaar HPF. Magnetic resonance imaging-assessed adipose tissue and serum lipid and insulin concentrations in growth hormone-deficient adults. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1995 **15** 1543–1548.
11 Alford F, Hew FL, Koschmann M, Cristopher M, Rantzau C & Ward G. Defects of glucose metabolism in growth hormone-deficient (GHD) adults. *Endocrinology and Metabolism* 1994 **1** (Suppl B) 27.
12 O'Neal DN, Kalfas A, Dunning PL, Christopher MJ, Sawyer SD, Ward GM *et al.* The effect of 3 months of recombinant human growth hormone (GH) therapy on insulin and glucose-mediated glucose disposal and insulin secretion in GH-deficient adults: a minimal model analysis. *Journal of Clinical Endocrinology and Metabolism* 1994 **79** 975–983.
13 Hew FL, Koschmann M, Christopher M, Rantzau C, Vaag A, Ward G *et al.* Insulin resistance in growth hormone-deficient adults: defects in glucose utilization and glycogen synthase activity. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 555–564.
14 Whitehead HM, Boreham C, McIlrath EM, Sheridan B, Kennedy L, Atkinson AB *et al.* Growth hormone treatment of adults with growth hormone deficiency: results of a 13-month placebo controlled cross-over study. *Clinical Endocrinology* 1992 **36** 45–52.
15 Beshyah SA, Freemantle C, Shahi M, Anyaoku V, Merson S, Lynch S *et al.* Replacement treatment with biosynthetic growth hormone in growth hormone-deficient hypopituitary adults. *Clinical Endocrinology* 1995 **42** 73–84.
16 Amato G, Carella C, Fazio S, La Montagna G, Cittadini A, Sabatini D *et al.* Body composition, bone metabolism, and heart structure and function in growth hormone (GH)-deficient adults before and after GH replacement therapy at low doses. *Journal of Clinical Endocrinology and Metabolism* 1993 **77** 1671–1676.
17 Nass R, Huber RM, Klauss V, Muller OA, Schopohl J & Strasburger CJ. Effect of growth hormone (hGH) replacement therapy on physical work capacity and cardiac and pulmonary function in patients with hGH deficiency acquired in adulthood. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 552–557.

18 Rutherford OM, Beshyah SA, Scon J, Watkins Y & Johnston DG. Contractile properties of the quadriceps muscle in growth hormone-deficient hypopituitary adults. *Clinical Science* 1995 **88** 67–71.
19 Valcavi R, Gaddi O, Zini M, Iavicoli M, Mellino U & Portioli I. Cardiac performance and mass in adults with hypopituitarism: effects of one year of growth hormone treatment. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 659–666.
20 McGauley GA. Quality of life assessment before and after growth hormone treatment in adults with growth hormone deficiency. *Acta Pediatrica Scandinavica* 1989 **356** 70–72.
21 Mardh G, Lundin K, Borg G, Jonsson B & Lindeberg A. Growth hormone replacement therapy in adult hypopituitary patients with growth hormone deficiency: combined data from 12 European placebo-controlled clinical trials. *Endocrinology and Metabolism* 1994 **1** (Suppl A) 43–49.
22 Attanasio AF, Lamberts WJS, Matranga MCA, Birkett MA, Bates PC & Valk NK. Adult growth hormone (GH)-deficient patients demonstrate heterogeneity between childhood onset and adult onset before and during human GH treatment. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 82–88.
23 Yamasaki Y, Kawamori R, Matsushima H, Nishizawa H, Kodama M, Kajimoto Y *et al.* Atherosclerosis in carotid artery of young IDDM patients monitored by ultrasound high-resolution B-mode imaging. *Diabetes* 1994 **43** 634–639.
24 Irie M, Shizume K, Takano K, Kato Y, Tanaka T & Chihara K. Growth hormone replacement therapy in adults with growth hormone deficiency: a double blind, placebo-controlled cross-over trial in Japan. *Endocrinology and Metabolism* 1995 **2** 17–23.
25 Powrie J, Weissberger A & Sönksen P. Growth hormone replacement therapy for growth hormone-deficient adults. *Drugs* 1995 **49** 656–663.
26 Pignoli P, Tremoli E, Poli A, Oreste P & Paoletti R. Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. *Circulation* 1986 **74** 1399–1406.
27 Bonithon-Kopp C, Touboul P-J, Berr C, Leroux C, Mainard F, Courbon D *et al.* Relation of intima-media thickness to atherosclerotic plaques in carotid arteries. The vascular aging (EVA) study. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1996 **16** 310–316.
28 Tsukahara H, Gordienko DV, Tonshoff B, Gelalto MC & Goligorsky MS. Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney International* 1994 **45** 598–604.
29 Böger RH, Skamira C, Bode-Böger SM, Brabant G & Mühlen A. Nitric oxide may mediate hemodynamic effects of recombinant growth hormone in patients with acquired growth hormone deficiency. A double-blind, placebo-controlled study. *Journal of Clinical Investigation* 1996 **98** 2706–2713.

Received 3 April 2002
Accepted 11 December 2002

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Life Sciences 72 (2003) 2517–2524
Life Sciences
www.elsevier.com/locate/lifescie

The role of circulating ghrelin in growth hormone (GH) secretion in freely moving male rats

Yasuhiko Okimura[a,*], Kiyoharu Ukai[b], Hiroshi Hosoda[c], Masahiro Murata[d], Genzo Iguchi[d], Keiji Iida[d], Hidesuke Kaji[e], Masayasu Kojima[f], Kenji Kangawa[c], Kazuo Chihara[d]

[a] *Department of Basic Allied Medicine, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma, Kobe 654-0142, Japan*
[b] *Development Research Laboratories, Kaken Pharmaceutical Company, 14 Shinomiya, Minamikawara-cho, Yamashina Kyoto 607-8042, Japan*
[c] *Department of Biochemistry, National Cardiovascular Disease Center Research Institute, Fujishirodai, suita 565-8565, Japan*
[d] *Division of Endocrinology/Metabolism, Neurology, and Hematology/Oncology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, chuo-ku, Kobe 650-0017, Japan*
[e] *Department of Physiology and Biochemistry, College of Nursing Art and Culture, Hyogo, 13-71 Kitaoji-cho, Akashi 673-8588, Japan*
[f] *Division of Molecular Genetics, Institute of Life Sciences, Kurume University, Kurume, Fukuoka 839-0861, Japan*

Received 30 July 2002; accepted 4 December 2002

Abstract

To examine the physiological significance of plasma ghrelin in generating pulsatile growth hormone (GH) secretion in rats, plasma GH and ghrelin levels were determined in freely moving male rats. Plasma GH was pulsatilely secreted as reported previously. Plasma ghrelin levels were measured by both N-RIA recognizing the active form of ghrelin and C-RIA determining total amount of ghrelin. Mean ± SE plasma ghrelin levels determined by N-RIA and C-RIA were 21.6 ± 8.5 and 315.5 ± 67.5 pM, respectively, during peak periods when plasma GH levels were greater than 100 ng / ml. During trough periods when plasma GH levels were less than 10 ng / ml, they were 16.5 ± 4.5 and 342.1 ± 29.8 pM, respectively. There were no significant differences in plasma ghrelin levels between two periods. Next, effect of a GH secretagogue antagonist, [D-Lys-3]-GHRP-6, on plasma GH profiles was examined. There were no significant differences in both peak GH levels and area under the curves of GH (AUCs) between [D-Lys-3]-GHRP-6-treated and control rats. These findings

* Corresponding author. Tel.: +81-78-796-4540; fax: +81-78-796-4540.
E-mail address: okimura@ams.kobe-u.ac.jp (Y. Okimura).

doi:10.1016/S0024-3205(03)00147-4

suggest circulating ghrelin in peripheral blood does not play a role in generating pulsatile GH secretion in freely moving male rats.

Keywords: Ghrelin; GH rhythm; Freely-moving rats

Introduction

Growth hormone (GH) is episodically secreted in 3-hours intervals in freely moving conscious rats. The pulsatile GH secretion is mainly governed by two hypothalamic hormones, GH-releasing hormone (GHRH) and somatostatin [1]. GHRH appears to be essential in generating GH surges since passive immunizaton with anti-rat GHRH serum resulted in a complete elimination of GH surges [2]. On the other hand, somatostatin plays an important role in suppression of GH release during a trough period of pulsatile GH secretion [3]. GH surges generated by GHRH enhance somatostatin secretion, which in turn inhibits GH secretion [2]. As a result, GH is periodlically secreted with a cycle of 3 hours in freely moving rats.

In addition to GHRH, another GH-releasing peptide, ghrelin has been identified [4]. Ghrelin is a natural ligand for GH secretagogue (GHS) recetpors [5] which bind to GHS, synthetic compounds stimulating GH secretion [6,7]. Ghrelin stimulates GH secretion from cultured rat pituitary cells [4] and perifused rat pituitaries [8] and intravenously administered ghrelin stimulates plasma GH levels in humans [9,10] and rats [4,11]. In peripheral blood, ghrelin is detected using spscific RIA and main source of circulating ghrelin appears to be stomach since the resection of stomach markedly decreased plasma ghrelin levels [12]. The plasma ghrelin levels determined by RIA recognizing C-terminal portion of ghrelin were 220 and 130 pM in rats and humans, respectively [13,14]. However, RIA recoginizing n-octanoyl-ghrelin, the active form of ghrelin, revealed that bioactive form of ghrelin levels were 4 pM in rat plasma [13], which seems not so high as the ghrelin levels stimulating GH release from the cultured pituitary cells [4] or perifused rat pituitaries [8]. Thus, the physiological role of circulating ghrelin in stimulating GH secretion remains unknown. In the present study, we determined plasma ghrelin levels in freely moving rats and compared the levels in peak and trough GH periods to clarify a role of ghrelin in stimulating GH release. Furthermore, the effect of a GHS antagosist, [D-Lys-3]-GHRP-6 on a pulsatile GH secretion was examined.

Methods

Animals

Eight week-old male rats of Wister strain (Japan CLEA Inc., Osaka, Japan) were housed in an air-conditioned room at 22 ± 2 with a 12:12h light-dark cycle (light on at 06:00 h) and provided with laboratory chow (Oriental Yeast Co., Tokyo, Japan) and water ad libitum.

Experimental Procedure in Freely Moving, Conscious Rats

Under pentobarbital anesthesia (4.0 mg/100 g body weight, i.p.), an indwelling catheter was inserted through a jugular vein into the right atrium in each rat as described previously [2]. The

animals were then transferred to individual cages and were habituated to frequent handling as well as to the test environment to exclude any nonspecific effects of stress. One week after the catheterization, a small heparinized syringe was connected to extention tubing attached to the atrial cannula in the early morning on the day of the experiments. Blood samples were drawn every 30 min for 6 h (10:30 – 16:30) or for 7 h 30 min (9:00 – 16:30), immediately centrifuged, and plasma was separated and stored at −20 C until assayed for GH. The red blood cells were resuspended in physiological saline and returned to the rats after removal of the next blood sample.

Rat GH-RIA

Plasma rat GH levels were determined by RIA using NIDDK-rGH-RP-2 as a reference preparation. The intra- and inter-assay coefficients of variation were 7.4 and 8.0 %, respectively.

Rat ghrelin-RIA

Blood samples were collected at 11:00, 13:30 and 15:30 through indwelling catheters from freely moving rats, added EDTA·2Na (1 mg/ml blood) and aprotinin (500 U/ml), and centrifuged. After the plasma had been diluted to one half with 0.9% saline, it was applied to a Sep-Pak C_{18} cartridge preequilibrated with 0.9% saline. The cartridge was washed with saline and 10 % CH_3CN solution containing 0.1% TFA. Adsorbed peptides were eluted with 60 % CH_3CN solution containing 0.1% TFA, lyophilized, then subjected to ghrelin RIAs.

To determine plasma ghrelin levels, two rat Ghrelin RIAs were used: N-RIA and C-RIA. N-RIA was performed using the antiserum against rat ghrelin-(1-11) and C-RIA was performed using the antiserum against rat ghrelin [13–28]. The antiserum against rat ghrelin-(1-11) specifically recognizes the n-octanoylated portion at Ser3 of ghrelin and plasma ghrelin levels determined by N-RIA indicate active form of ghrelin as described previously [13]. The antiserum against rat ghrelin-(13–28) equally recognizes n-octanoyl-modified and des-n-octanoylated ghrelins, and C-RIA determines whole amount of ghrelin. The methods of N-RIA and C-RIA were described previously [13]. Briefly, synthetic rat [Tyr^{29}]ghrelin-(1-28) and [Tyr^{0}]ghrelin-(13-28) were radioiodinated by the lactoperoxidase method and used as a tracer in N-RIA and C-RIA, respectively. A diluted sample or a standard peptide solution (100 μl) was incubated with 200 μl of antiserum diluted with RIA buffer containing 50 mM sodium phosphate (pH 7.4), 0.5% BSA, 80 mM NaCl, 25 mM EDTA·2Na, 0.05% NaN_3, 0.5% Triton X-100 and 0.5 % normal rabbit serum for 12 h. Then, the tracer solution was added and incubated for further 36 h. The bound and free tracers were separated by adding 100 μl of anti-rabbit IgG goat serum. All procedures were performed at 4 C. Samples were assayed in duplicate.

Repetitive administration of [D-Lys-3]-GHRP-6 in freely moving rats

A specific antagosist for GHS receptors, [D-Lys-3]-GHRP-6 [4], was used for blocking the effect of circulating ghrelin. Blood samples were drawn every 30 min for 7 h30 min (9:00 – 16:30) from freely moving rats, immediately centrifuged and plasma was separated and stored at −20 C until assayed for GH. In the rats receiving [D-Lys-3]-GHRP-6, 750 ng [D-Lys-3]-GHRP-6

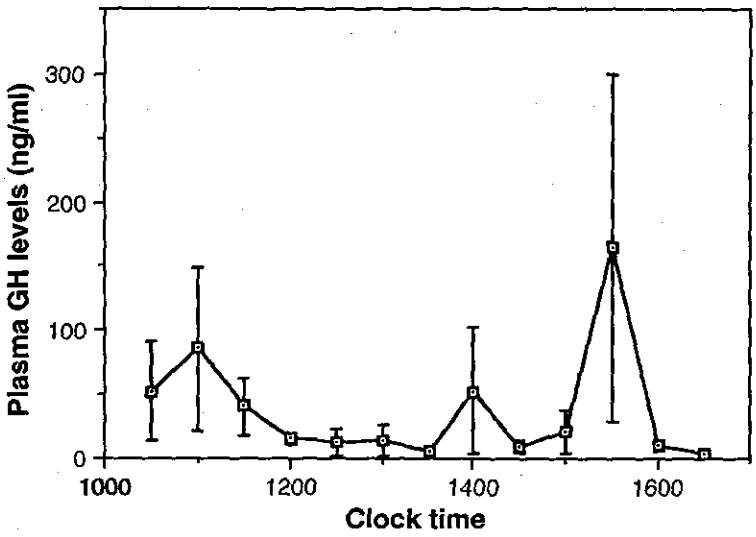

Fig. 1. Pulsatile GH secretion in freely moving male rats (n = 6).

was i.v. injected just after each blood sampling after the 9 th sampling at 13:00. In the control group, same amount of normal saline was injected.

Statistical analysis

The data were expressed as the mean ± SE. Statistics were analyzed by Mann-Whitney's U analysis.

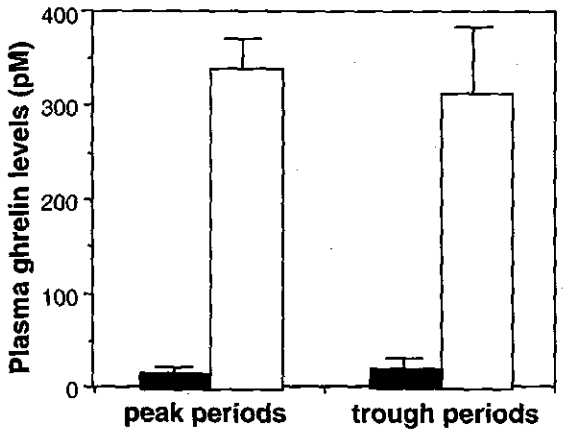

Fig. 2. The mean ± SE plasma ghrelin levels during peak and trough periods of GH secretion in freely moving male rats. Plasma ghrelin levels detemined by N-RIA (solid column) and C-RIA (open column) indicate the concentrations of active form of ghrelin and total ghrelin, respectively. There were no significant differences in plasma ghrelin levels between peak and trough periods of GH irrespective of RIA method.

Results

Plasma ghrelin and GH levels in freely moving rats

Normal GH profiles are shown in Fig. 1. Plasma GH, sampled every 30 min during 6 h, exhibited pulsatile variations. The mean ± SE plasma ghrelin levels determined by N-RIA and C-RIA were 21.6 ± 8.5 and 315.5 ± 67.5 pM, respectively when plasma GH levels were greater than 100 ng / ml. The mean ± SE plasma ghrelin levels determined by N-RIA and C-RIA were 16.5 ± 4.5 and 342.1 ± 29.8 pM, respectively when plasma GH levels were less than 10 ng / ml (Fig. 2). There were no significant differences in plasma ghrelin levels between the two groups.

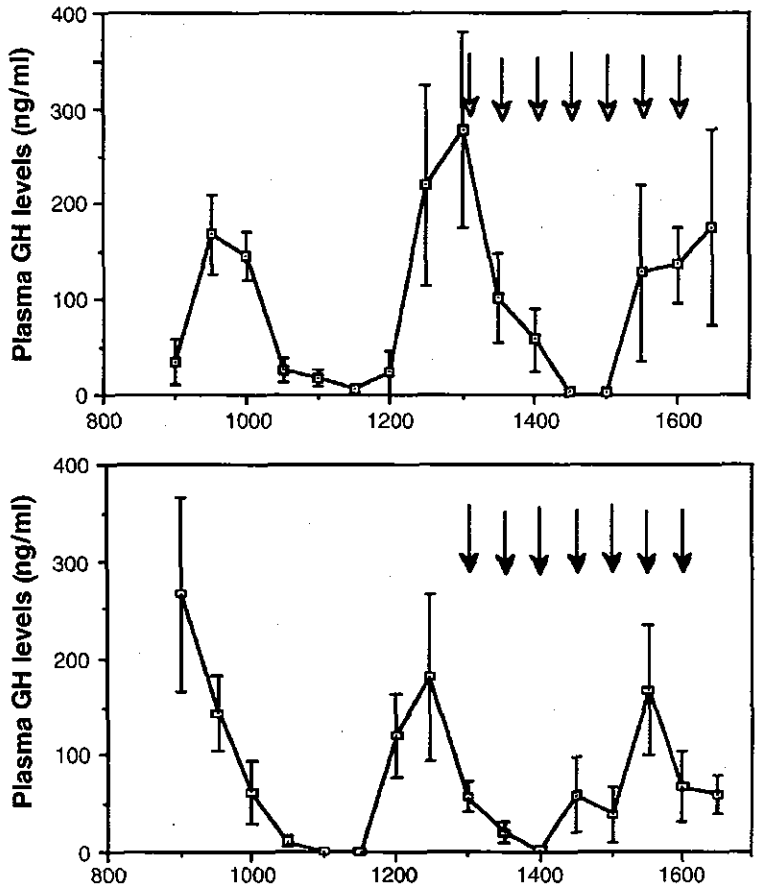

Fig. 3. Effect of [D-Lys-3]-GHRP-6 on plasma GH profiles in freely moving rats.750 ng [D-Lys-3]-GHRP-6, a specific antagosist for GHS receptors, was i.v. injected (solid arrow, n = 8) just after each blood sampling after the 9 th sampling at 13:00 (lower panel). In the control group, same volume of vehicle was injected (open arrow, upper panel, n = 8). In [D-Lys-3]-GHRP-6-treated rats, pulsatile GH secretion was observed after [D-Lys-3]-GHRP-6 treatment as well as before treatment.

Table 1
Effects of repetitive administration of [D-Lys-3]-GHRP-6 on plasma GH profiles in freely moving rats

	Before administration		After administration	
	Peak GH levels (ng/ml)	AUC (ng· min/ml)	Peak GH levels (ng/ml)	AUC (ng· min/ml)
[D-Lys-3]-GHRP-6 (n = 8)	341 ± 75	17089 ± 2935	357 ± 64	17672 ± 2101
normal saline (n = 8)	310 ± 69	19796 ± 4092	469 ± 96	23415 ± 4024

Data represent the peak GH and AUC (area under the curve) before and after administration of [D-Lys-3]-GHRP-6.

Effect of [D-Lys-3]-GHRP-6 on plasma GH profiles in freely moving rats

In individual rats treated with [D-Lys-3]-GHRP-6, pulsatile GH secretion was observed after [D-Lys-3]-GHRP-6 treatment as well as before [D-Lys-3]-GHRP-6 treatment (Fig. 3).

Also, the AUCs before and after [D-Lys-3]-GHRP-6 treatment were not different in both groups (Table 1). Since the GH peak occurred at various time points in individual rats, mean ± SE maximal GH concentrations after 13:00 was compared in [D-Lys-3]-GHRP-6-treated and control groups. They were 357 ± 64 ng / ml, 469 ± 96 ng / ml, respectively; also no significant differences were observed.

Discussion

In the present study, plasma ghrelin levels were not remarkably changed whereas GH was pulsatilely secreted in freely moving rats. This finding suggests that circulating ghrelin is not a main regulator for GH rhythm. In agreement with this finding, we have already reported that intravenous administration of GHRH antibody completely abolishes pulsatile GH release in freely moving rats [2]. However, in patients with an inactivating defect of GHRH receptor, very small pulsatile GH releases were observed using a sensitive fluoroimmunosassay [15,16]. In our previous study in which GHRH antibody completely abolished GH rhythm in freely moving rats, small GH pulse may not have been detected since RIA used in the study is less sensitive than the fluoroimmunosassay. Therefore, the possibilities that factors other than GHRH might trigger pulsatile GH releases and that GHRH might enhance it are not excluded.

It has recently been reported that ghrelin is also secreted pulsatilely [17,18]. However, Tolle et al reported that the releases of ghrelin and GH were not correlated, although the secretion of ghrelin has rhythm. In the present study, the varying range of active form of ghrelin determined by N-RIA was 7.9 – 45.6 pM; the variances in ghrelin levels were not so much as those of GH. Plasma ghrelin levels during GH peak periods were not different from those during trough periods. Furthermore, the plsma ghrelin levels were less than the levels required for stimulating GH secretion from cultured rat pituitary cells [4] or perifused rat pituitaries [8]. These findings suggest that circulating ghrelin does not play a role in generating pulsatile GH secretions.

To further clarify the issue that circulating ghrelin plays a role in GH release, we repetitively administered a GHS antagosist, [D-Lys-3]-GHRP-6 to freely moving rats and examined its effect on GH secretion. We could not find that [D-Lys-3]-GHRP-6 reduced plasma GH levels and AUCs. This also suggests that circulating ghrelin is not involved in generating pulsatile GH secretion. However, these findings do not exclude the possibility that ghrelin in the hypothalamus is involved in it.

Conclusion

Plasma ghrelin levels were not so changed as plasma GH levels, and there were no significant differences in plasma ghrelin levels between peak and trough GH periods. Repetitive administrations of [D-Lys-3]-GHRP-6 did not affect GH secretion. These findings suggest plasma ghrelin does not play a pivotal role in the pulsatile release of GH.

Acknowledgements

This study was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare; and The Foundation for Growth Science.

References

[1] Giustina A, Veldhuis JD. Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. Endocr Rev 1998;19(6):717–97.
[2] Sato M, Chihara K, Kita T, Kashio Y, Okimura Y, Kitajima N, Fujita T. Physiological role of somatostatin-mediated autofeedback regulation for growth hormone: importance of growth hormone in triggering somatostatin release during a trough period of pulsatile growth hormone release in conscious male rats. Neuroendocrinology 1989;50(2):139–51.
[3] Tannenbaum GS, Ling N. The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. Endocrinology 1984;115(5):1952–7.
[4] 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(6762):656–60.
[5] Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchet AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 1996;273(5277):974–7.
[6] Bowers CY, Momany FA, Reynolds GA, Hong A. On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 1984;114(5):1537–45.
[7] Patchett AA, Nargund RP, Tata JR, Chen MH, Barakat KJ, Johnston DB, Cheng K, Chan WW, Butler B, Hickey G, Jacks T, Schlem K, Pong SS, Chaunt L-YP, Chen HY, Frazier E, Leung KH, Chiu S-HL, Smith RG. Design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue. Proc Natl Acad Sci U S A 1995;92(15):7001–5.
[8] Yamazaki M, Nakamura K, Kobayashi H, Matsubara M, Hayashi Y, Kangawa K, Sakai T. Regulational effect of ghrelin on growth hormone secretion from perifused rat anterior pituitary cells. J Neuroendocrinol 2002;14(2):156–62.
[9] Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 2000;85(12):4908–11.
[10] Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C, Casanueva FF. Ghrelin-induced growth hormone secretion in humans. Eur J Endocrinol 2000;143(6):R11–4.
[11] Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J, Bluet-Pajot MT. In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. Neuroendocrinology 2001;73(1):54–61.
[12] Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Ntsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nkao K. Stomach is a major source of circulating ghrelin,

and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 2001;86(10):4753–8.
[13] Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000;279(3):909–13.
[14] Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S. Plasma Ghrelin Levels in Lean and Obese Humans and the Effect of Glucose on Ghrelin Secretion. J Clin Endocrinol Metab 2001;87(1):240–4.
[15] Roelfsema F, Biermasz NR, Veldman RG, Veldhuis JD, Frolich M, Stokvis-Brantsma WH, Wit JM. Growth hormone (GH) secretion in patients with an inactivating defect of the GH-releasing hormone (GHRH) receptor is pulsatile: evidence for a role for non-GHRH inputs into the generation of GH pulses. J Clin Endocrinol Metab 2001;86(6):2459–64.
[16] Maheshwari HG, Pezzoli SS, Rahim A, Shalet SM, Thorner MO, Baumann G. Pulsatile growth hormone secretion persists in genetic growth hormone-releasing hormone resistance. Am J Physiol Endocrinol Metab 2002;282(4):E943–51.
[17] Bagnasco M, Kalra PS, Kalra SP. Ghrelin and leptin pulse discharge in fed and fasted rats. Endocrinology 2002; 143(2):726–9.
[18] Tolle V, Bassant M, Zizzari P, Poindessous-Jazat F, Tomasetto C, Epelbaum J, Bluet-Pajot M. Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. Endocrinology 2002;143(4): 1353–61.

European Journal of Endocrinology (2004) 151 447–450 ISSN 0804-4643

CLINICAL STUDY

Intravenous administration of ghrelin stimulates growth hormone secretion in vagotomized patients as well as normal subjects

Ryoko Takeno[1], Yasuhiko Okimura[2], Genzo Iguchi[1], Masahiko Kishimoto[1], Takumi Kudo[1], Kentaro Takahashi[1], Yutaka Takahashi[1], Hidesuke Kaji[3], Masakazu Ohno[4], Hajime Ikuta[4], Yoshikazu Kuroda[4], Tetsuji Obara[5], Hiroshi Hosoda[6], Kenji Kangawa[6] and Kazuo Chihara[1]

[1]*Division of Endocrinology/Metabolism, Neurology and Hematology/Oncology, Department of Clinical Molecular Medicine and* [4]*Division of Gastroenterological Surgery, Kobe University Graduate School of Medicine, Hyogo, 650-0017, Japan,* [2]*Department of Basic Allied Medicine, Kobe University School of Medicine, Kobe, 654-0142,* [3]*College of Nursing Art and Science, Hyogo, 673-8588,* [5]*Obara Hospital, Hyogo, 654-0121 and* [6]*Department of Biochemistry, National Cardiovascular Center, Research Institute, Osaka, 565-8565, Japan*

(Correspondence should be addressed to Yasuhiko Okimura, Department of Basic Allied Medicine, Kobe University School of Medicine, 7-10-2, Tomogaoka, Suma-ku, Kobe, 654-0142, Japan; Email: okimura@ams.kobe-u.ac.jp)

Abstract

Objective: Ghrelin is a potent peptide stimulating GH secretion. Besides its direct action on the pituitary, ghrelin has been reported to stimulate GH release via the vagal afferent nerve in rats. To examine the involvement of vagal nerve in ghrelin-induced GH secretion in humans, GH responses to ghrelin were compared between vagotomized patients with gastrectomy and normal subjects.

Methods: Ghrelin (0.2 μg/kg) or GHRH (1 μg/kg) was administered intravenously in vagotomized patients and normal subjects on separate days, and plasma GH responses to the stimuli were examined.

Results: Ghrelin caused a significant plasma GH rise in both vagotomized patients and normal subjects. Peak GH levels in vagotomized patients (37.5±16.9 ng/ml) were not different from those in normal subjects (29.9±23.1 ng/ml). The areas under the curve of GH response to ghrelin did not differ between the two groups. GHRH also increased GH levels, and peak GH levels and areas under the curve after GHRH stimulation were also comparable between vagotomized patients and normal subjects.

Conclusions: In the present study, the involvement of the afferent vagal nerve in ghrelin-induced GH secretion was not confirmed in humans.

European Journal of Endocrinology **151** 447–450

Introduction

Growth hormone (GH) secretion is governed by two hypothalamic hormones, growth hormone-releasing hormone (GHRH) and somatostatin. GHRH stimulates the secretion of GH while somatostatin inhibits it (1). In addition to GHRH, another GH-releasing peptide, ghrelin, has been identified (2). Ghrelin is a natural ligand for the receptors for GH secretagogues, synthetic compounds with GH-releasing activity (3). Ghrelin stimulates GH secretion from cultured rat pituitary cells (2), and intravenously administered ghrelin increases plasma GH levels in humans (4–6) and rats (2, 7). In addition, ghrelin stimulates food intake when administered intraventricularly or intravenously (8, 9). Ghrelin is detected in peripheral blood, and the main source of circulating ghrelin appears to be the stomach since resection of stomach was found to decrease plasma ghrelin levels (10). However, circulating ghrelin is unlikely to play a role in regulating GH secretion since pulsatile GH secretion was preserved in freely moving rats treated with GH secretagogue blocker (11). On the other hand, gastric afferent vagal nerve has been reported to be involved in stimulatory effects of ghrelin on GH secretion and food intake in rats (9, 12). These findings led us to compare the GH responses to ghrelin injections in patients with vagotomy and normal healthy subjects.

Materials and methods

Subjects

Six vagotomized patients associated with gastrectomy and six healthy volunteers were studied. Four patients have undergone total gastrectomy and two patients have undergone partial gastrectomy, with the fundus of the stomach remaining, as a result of stomach cancer and have no recurrence. All the patients have undergone truncal vagotomy and have no recurrence. Clinical characteristics of participants were summarized in Table 1. All subjects gave their written informed

Table 1 Clinical characteristics of six patients with vagotomy associated with gastrectomy and six normal subjects. All values are mean±S.D.

	Vagotomized patients	Normal subjects	*P* value
Number	6	6	N.D.
Age (years)	56.8±14.4	33.8±7.9	$P<0.05$
Sex (female/male)	2/4	1/5	N.D.
Body mass index (kg/m^2)	19.7±1.5	21.7±4.3	$P=0.09$
IGF-I (ng/ml)	114±17.3	218±59.1	$P<0.05$
PG (mg/dl)	87.8±11.5	90.4±16.7	$P=0.495$
Total ghrelin (fmol/ml)	93.6±26.5	280.9±120.7	$P<0.05$
Period after gastrectomy (years)	3.2±3.0		

PG, plasma glucose. N.D., not different.

consent to participate in the study, which was approved by the Kobe University Ethical Committee.

Ghrelin test and GHRH test

All vagotomized patients and normal subjects underwent a ghrelin test, and five vagotomized patients and six normal subjects underwent a GHRH test on separate days. After an overnight fast, at 0830–0900 h an indwelling catheter was placed into an antecubital vein of the forearm. Blood samples were obtained just before and 15, 30, 45, 60, 90 and 120 min after the bolus injection of human ghrelin (0.2 μg/kg body weight) or human GHRH1-40 (1 μg/kg). Blood samples were centrifuged and the resulting plasma was stored at −20 °C until assay. Plasma GH concentrations were determined by ELISA (Toso, Tokyo, Japan). The sensitivity of the assay was 0.15 ng/ml. The inter- and intra-assay coefficients of variation were 2.9 and 2.4%, respectively. The GH responses are expressed in absolute terms and as areas under curves (AUCs) calculated by trapezoidal integration. Plasma insulin-like growth factor (IGF)-I levels were determined by immunoradiometric assay (Daiichi Radio Isotope, Tokyo, Japan). The sensitivity of the assay was 4.2 ng/ml. The inter- and intra-assay coefficients of variation were 3.0 and 3.5%, respectively. Total plasma ghrelin levels were determined with RIA recognizing C-terminal ghrelin (13). Plasma glucose levels were measured by glucose oxidase colorimetric method (Sanwa Kagaku, Nagoya, Japan).

Statistical analysis

The statistical analysis was carried out using nonparametric ANOVA (Friedman test) and then Wilcoxon test, as appropriate. The results are expressed as mean±S.E.M. $P < 0.05$ was considered significant.

Results

Intravenous administrations of ghrelin caused significant plasma GH rises with peak levels of 37.5±16.9 and 29.9±23.1 ng/ml in vagotomized patients with gastrectomy and normal subjects, respectively. AUCs after ghrelin administration were 2037.1±938.0 and 1455.9±856.9 ng/min per ml in the patients and normal subjects, respectively. Peak GH levels and AUCs did not differ between two groups (Fig. 1). GHRH administration also elevated plasma GH levels with peak levels of 16.5±7.0 and 9.4±1.8 ng/ml in

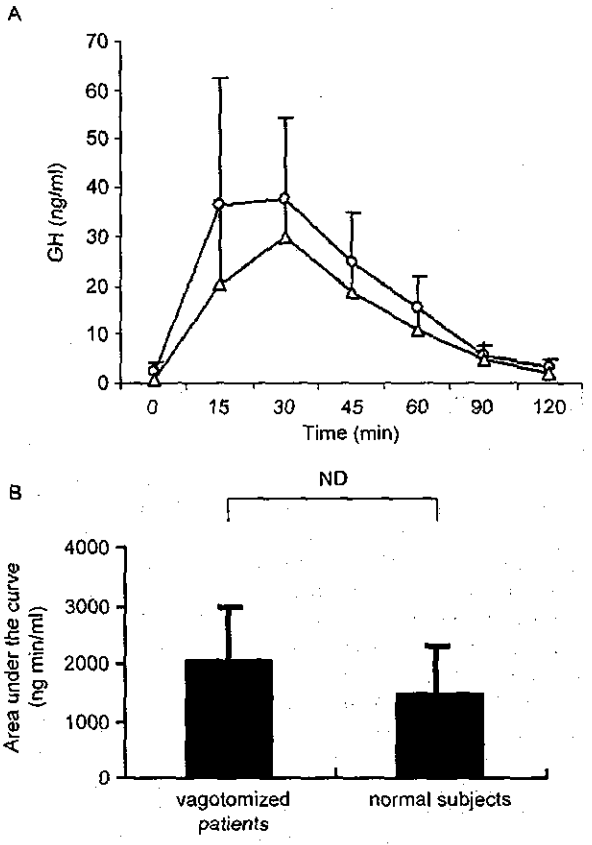

Figure 1 A. Effect of intravenous administration of ghrelin (0.2 μg/kg) on plasma GH levels (mean±S.E.M.) in six vagotomized patients ○ and six normal subjects △. B. AUCs (mean±S.E.M.) in response to ghrelin are shown in vagotomized patients and normal subjects.

vagotomized patients and normal subjects, respectively. AUCs after GHRH injection were 1210.0±641.6 and 686.6±101.2 ng/min per ml in the patients and normal subjects, respectively. There was no difference in GHRH-induced GH response between vagotomized patients and normal subjects (Fig. 2). Peak GH levels to ghrelin were greater than those to GHRH in the vagotomized patients. Body mass index and fasting plasma glucose were not different in both groups, but plasma IGF-I and basal ghrelin levels were lower in the patients than in normal subjects (Table 1).

Discussion

Asakawa *et al.* (9) have reported that ghrelin exhibited potent orexigenic effects which is lost after vagotomy in rats. In addition, Date *et al.* (12) have shown that GH responses to ghrelin were attenuated in vagotomized rats compared with control rats. Also, they have shown the presence of ghrelin receptor in vagal afferent nerve and the attenuation of ghrelin-induced c-Fos expression in neuropeptide Y-producing and GHRH-producing neurons in the hypothalamus of vagotomized rats. Furthermore, Sakata *et al.* (14) reported recently, having used retrograde tracing with fluorogold, that some cells containing ghrelin receptors in the nodose ganglion project to the stomach. These findings suggest a possible mechanism for ghrelin produced in the stomach to stimulate GH release and food intake via vagal afferent nerves in rats. In agreement with these results that the central nervous system, at least in part, is involved in ghrelin action, Popovic *et al.* (15) have reported that the main action of ghrelin to stimulate GH release is exerted at the hypothalamic level.

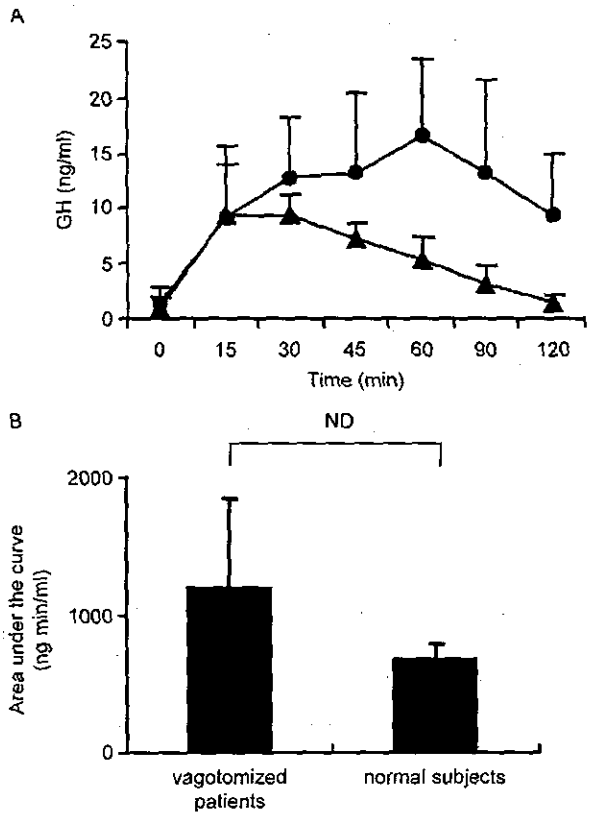

Figure 2 A. Effect of intravenous administration of GHRH (1 μg/kg) on plasma GH levels (mean±S.E.M.) in six vagotomized patients ● and six normal subjects ▲. B. AUCs (mean±S.E.M.) in response to GHRH are shown in vagotomized patients and normal subjects.

In contrast, in the present study, intravenous administration of ghrelin resulted in a marked GH release in both vagotomized patients and normal subjects to a similar extent, and GH secretion to ghrelin was greater than to GHRH. These findings suggest no involvement of the vagal nerve in ghrelin-induced GH secretion in humans.

Although the reason why GH secretions in response to ghrelin differed between humans and rats is unclear, several possibilities may explain it. One possibility is a modulation of GH response by IGF-I. It is well known that IGF-I has an inhibitory action on pulsatile and provocation-induced GH secretions in humans (16, 17). Since plasma IGF-I levels in the patients with vagotomy were lower than those in normal subjects, it may be responsible for the augmentation of GH response to ghrelin in vagotomized patients. However, not all the provocative tests were influenced by IGF-I. For example, IGF-I is unlikely to reduce GH responses to arginine. A s.c. administration of IGF-I does not suppress GH response to i.v. infusion of arginine (18). Thus, it is unclear that IGF-I, in fact, suppresses GH release to ghrelin, although the possibility that IGF-I modulates GH response to ghrelin cannot be excluded.

The difference in mean age between the vagotomized patients and normal subjects may have affected GH responses. In general, GH responses to provocative stimuli are decreased in the elderly. Broglio *et al.* (19) have reported the attenuated GH secretion to ghrelin in elderly people. Hence, the GH response to ghrelin in the vagotomized patients may be underestimated but not overestimated compared with normal subjects since the vagotomized patients were older than normal subjects. Therefore, it is unlikely that the attenuated responses to ghrelin, which should have been observed in vagotomized patients as well as vagotomized rats, were masked in the vagotomized patients because of their age.

Gastrectomy itself may influence GH secretion induced by ghrelin in humans. In rats, however, resection of the gastrointestinal tract with vagotomy also leads to attenuated GH secretion to growth hormone-releasing peptide-6 (GHRP-6), one of the GH secretagoues, which is comparable to the findings in rats with selective vagotomy (20). Therefore, gastrectomy itself