Plasma ghrelin levels in healthy elderly volunteers: the levels of acylated ghrelin in elderly females correlate positively with serum IGF-I levels and bowel movement frequency and negatively with systolic blood pressure

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Abstract

Aging is associated with a decrease in growth hormone (GH) secretion, appetite and energy intake. As ghrelin stimulates both GH secretion and appetite, reductions in ghrelin levels may be involved in the reductions in GH secretion and appetite observed in the elderly. However, only preliminary studies have been performed on the role of ghrelin in elderly subjects. In this study, we sought to clarify the physiologic implications of the age-related alterations in ghrelin secretion by determining plasma ghrelin levels and other clinical parameters in healthy elderly subjects. Subjects were ≥ 65 years old, corresponding to the SENIEUR protocol, had not had a resection of the upper gastrointestinal tract and had not been treated with hormones. One hundred and five volunteers (49 men and 56 women) were admitted to this study ($73{\cdot}4 \pm 6{\cdot}3$ years old). Plasma levels of acylated ghrelin in elderly female subjects positively correlated with serum IGF-I levels and bowel movement frequency and negatively with systolic blood pressure. In elderly men, desacyl ghrelin levels correlated only weakly with bowel movement frequency. These findings suggest that the plasma levels of the acylated form of ghrelin may influence the age-related alterations in GH/IGF-I regulation, blood pressure and bowel motility. These observational associations warrant further experimental studies to clarify the physiologic significance of these effects.

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Introduction

Aging is associated with progressive decreases in growth hormone (GH) secretion, appetite and energy intake (Wurtman *et al.* 1988, Corpas *et al.* 1993, Morley 1997, Muller *et al.* 1999). This reduced GH secretion is termed 'somatopause' and may be a cause of age-related metabolic and physiologic changes, including reduced lean body mass and expansion of adipose mass. Altered blood lipid profiles also favor the development of vascular diseases that may increase overall mortality. The age-related reduction in energy intake has been termed 'the anorexia of aging' and predisposes to the development of undernutrition (Morley 1997). Common in older people, undernutrition has been implicated in the development and progression of chronic diseases commonly affecting the elderly, as well as in increasing mortality (Wurtman *et al.* 1988).

The mechanisms underlying the reduced GH secretion in aged animals and humans are complex (Muller *et al.* 1999,

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Chapman 2000). Age-related changes appear to involve the function of hypothalamic peptides specifically regulating GH secretion, and GH-releasing hormone (GHRH) and somatostatin (SS), appear to play a major role in this event. Experimental evidence indicates that within the rat hypothalamus, GHRH synthesis is impaired with increased age; relative hyperfunction of the SS-ergic system is also found in this animal. The physiologic causes of the anorexia of aging are largely unknown and probably multifactorial (Morley 1997). Possible mechanisms include a reduction in the central and/or peripheral feeding drives and increased activity of central and/or peripheral satiety signals (Martinez *et al.* 1993, Morley 1997, de Jong *et al.* 1999).

Ghrelin, a 28-amino-acid peptide, exhibits a variety of actions, including vasorelaxation (Nagaya *et al.* 2001, Shimizu *et al.* 2003) and stimulation of GH secretion (Takaya *et al.* 2000, Arvat *et al.* 2001, Hataya *et al.* 2001), appetite (Korbonits *et al.* 2004, van der Lely *et al.* 2004) and gastrointestinal motility (Masuda *et al.* 2000, Trudel *et al.* 2002, Fujino *et al.* 2003). A portion of ghrelin possesses a unique fatty acid modification, *n*-octanoylation, at Ser 3 (Kojima *et al.* 1999). Of the two circulating forms of ghrelin, acylated and unacylated (desacyl), the acylated form is thought to be essential for ghrelin biologic activity. Recently, however, desacyl ghrelin was reported to influence both cell proliferation and adipogenesis (Cassoni *et al.* 2001, Bedendi *et al.* 2003, Broglio *et al.* 2004, Thompson *et al.* 2004), prompting us to hypothesize that alterations in ghrelin may be involved in the reduction of GH secretion and appetite in elderly subjects. Preliminary studies using small numbers of elderly subjects demonstrated that the mean plasma concentrations of total ghrelin in normal weight geriatric subjects were lower than those present in younger, normal-weight subjects (Rigamonti *et al.* 2002, Sturm *et al.* 2003). In addition, GH response to ghrelin administration in elderly subjects is lower than that seen in young subjects (Broglio *et al.* 2003). While ghrelin mRNA levels in the stomach gradually decrease with increasing age in rats, serum levels of total ghrelin did not exhibit obvious age-related variation (Liu *et al.* 2002). In contrast, studies in rat indicated that both stomach ghrelin secretion and ghrelin-induced GH secretion increased in aged rats in comparison to younger rats (Englander *et al.* 2004). Total ghrelin secretion also increases with aging in monkeys (Angeloni *et al.* 2004). Although the disparity between humans and other animal models may be due to species differences, the number of human subjects was rather small. In addition, no adjustment of plasma ghrelin levels by other parameters was attempted, leaving the results of these human studies in question.

In this study, we determined the plasma concentrations of the two ghrelin forms, acylated and desacyl ghrelin, and their relationship to various anthropometric, hormonal and metabolic parameters in 105 elderly volunteers. Using these measurements and appropriate analyses, we sought to clarify the age-related alteration in ghrelin secretion and the associated physiologic implications in elderly subjects.

Materials and Methods

Subjects

One hundred and thirty-seven (62 male and 75 female) elderly volunteers were registered for our study. Thirty-two that did not satisfy the criteria for this study were excluded. Finally, 105 (49 male and 56 female) volunteers aged 65–94 years were subjected to analysis. All of the subjects were Japanese, and were recruited from the outpatient clinics of Kyoto University Hospital ($n=66$ (male, $n=36$; female, $n=30$)) and Kyoto Preventive Medical Center ($n=39$ (male, $n=13$; female, $n=26$)). The inclusion criteria were as follows: 1. ≥ 65 years of age; 2. correspondence with the SENIEUR protocol (Ligthart *et al.* 1984); 3. provision of written consent to participate in this study. Patients with either past history of upper gastrointestinal tract resection or present use of either hormones or steroids were excluded. The SENIEUR protocol provides strict admission criteria for human immunogerontologic studies. This protocol used clinical information (infection, inflammation, malignancy and other conditions, including acute myocardial infarction, treated cardiac insufficiency, hypertension of arteriosclerotic or diabetic origin, dementia, pregnancy, malnutrition, alcoholism and drug abuse), laboratory data (erythrocyte sedimentation rate, hemoglobin levels, mean corpuscular volume, leukocyte count with differentiation, immunoelectrophoresis, urinalysis and serum concentrations of urea, alkaline phosphatase, glucose, ASAT, ALAT and protein) and pharmacologic interference (prescribed medication for the treatment of the disorders defined above, anti-inflammatory drugs, hormones and analgesics) (Ligthart *et al.* 1984). This study included two exclusion criteria (no past resection of the upper gastrointestinal tract and no current treatment with hormones or steroids) to optimize endocrinologic and metabolic examination of stomach-derived hormones. The subjects who met all criteria were recognized as healthy subjects. Younger subjects, in whom plasma ghrelin levels were used for comparison with those in elderly subjects, were described previously (Akamizu *et al.* 2005). They were 16 male and 20 female Japanese volunteers 21–61 years of age. None of the subjects suffered from any known medical conditions or were currently taking medication. The period of the study was from March to September 2004. The study protocol was approved by the ethics committees on human research of Kyoto University Graduate School of Medicine and Kyoto Preventive Medical Center. Written, informed consent was obtained from all subjects prior to enrollment.

Laboratory analyses and biomedical factors

Blood samples for hormone and glucose analyses were drawn from a forearm vein in the morning after overnight fast. Plasma samples were prepared as previously described (Kojima *et al.* 1999, Akamizu *et al.* 2005). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 500 kallikrein inactivator U/ml), were centrifuged at 4 °C. We added 1 N mol/l HCl (10% volume of plasma volume) to the separated plasma immediately. Plasma levels of acylated and unacylated ghrelin were measured with two commercially available ELISA kits, the Active Ghrelin ELISA and Desacyl-Ghrelin ELISA respectively, according to the manufacturer's protocol (Mitsubishi Kagaku Iatron, Tokyo, Japan) (Akamizu *et al.* 2005). The minimal detection limits for acylated and desacyl ghrelin in this assay system were 2·5 and 12·5 fmol/ml respectively. The intra- and interassay coefficients of variation were 6·5% and 9·8% for acylated ghrelin and 3·7% and 8·1% for desacyl ghrelin respectively. Ghrelin measurements of samples from the older and young subjects were performed with the same kits, but not in the same assay. Plasma glucose was measured by the glucose oxidase method. Serum GH, insulin-like growth factor (IGF)-I and insulin concentrations were measured by immunoradiometric assay (IRMA), while serum leptin levels were measured by RIA (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Insulin resistance was calculated according to the homeostasis model of assessment of insulin resistance (HOMA-IR), calculated as insulin (μU/ml) × blood glucose (mmol/l)/22·5 (Haffner *et al.* 1997).

The questionnaire presented to all subjects collected information about their sleeping time duration, bowel movements, smoking habits, alcohol consumption, use of medication, past medical history and physical activity. The question about bowel frequency was, 'How often do you usually defecate – once a day, more than once a day or once per 2 or 3 days?'

Statistical analysis

Data are expressed as the mean ± S.D. We used Student's *t*-test to compare the means of the variables measured in both groups. The relationships between ghrelin concentrations and the variables studied were assessed by multiple regression analysis. The variables examined in the multiple regression models were site of recruitment, gender, age or age group (elderly and younger group), body-mass index (BMI), sleeping duration and blood levels of GH, IGF-I, insulin, glucose and leptin. The associations between ghrelin concentrations and blood pressure or bowel movement were assessed by multiple regression analysis after adjustment by the potential confounding factors according to gender. As the ghrelin distribution was slightly skewed, natural logarithms of ghrelin were used for the regression analysis. To identify the subsets of parameters that are statistically significantly related to each hormone level, we performed multiple regression analysis with a backward-elimination procedure after adjustment for the potential effect of site. All statistical analyses were performed by SAS, Version 8·02 (SAS Institute, Cary, NC, USA). *P* values less than 0·05 were considered to be statistically significant.

Results

Plasma ghrelin concentrations in elderly subjects

We examined the anthropometric, hormonal and metabolic parameters of elderly volunteers (Table 1). The levels of acylated ghrelin in plasma were not significantly different between male and female subjects, while plasma levels of desacyl ghrelin in female subjects were significantly higher than those observed in male subjects (male, 53·3 ± 41·5 fmol/ml; female, 72·0 ± 46·1 fmol/ml; $P=0·031$). In comparison to our previous study of younger volunteers (mean age = 33·5 ± 9·0, $n=36$) (Akamizu *et al.* 2005), plasma levels of acylated ghrelin in elderly female subjects were significantly reduced from the levels in younger female subjects (11·9 ± 9·8 vs 19·9 ± 9·8 fmol/ml; $P=0·004$) (Fig. 1A and B). We did not observe any significant differences in the plasma levels of acylated ghrelin in men (9·3 ± 11·6 vs 10·9 ± 6·1 fmol/ml) or desacyl ghrelin levels in both sexes (male, 53·3 ± 41·5 vs 49·1 ± 23·5 fmol/ml; female, 72·0 ± 46·1 vs 79·8 ± 53·9 fmol/ml) between the elder and younger subjects. The ratios of acylated to desacyl ghrelin (A/D ratio) in elderly female subjects were significantly lower than those in younger female subjects (16·3 ± 8·2 vs 26·8 ± 7·8; $P=0·001$). Men did not exhibit any significant differences between the age groups (18·0 ± 11·0 vs 22·6 ± 8·8; $P=0·101$) (Fig. 1C). Age, BMI, insulin, leptin and HOMA-IR levels, however, also differed significantly between the sexes. In addition, the volunteers were recruited from two independent sites. Although nearly all of the parameters, including ghrelin levels, did not differ significantly between the sites, the A/D ratios in both sexes and diastolic blood pressure (BP) values in men were significantly different (Table 1). To account for these differences, recruitment site, gender and age group were included as independent variables in the multiple regression analyses.

Correlations of ghrelin concentrations with various parameters in elderly subjects

In contrast to the results in Student's *t*-test, plasma levels of acylated ghrelin in women were not correlated with age group in the multiple regression analyses ($P=0·914$) (Table 2), suggesting that those in elderly female subjects

Table 1 Characteristcs of elderly subjects and their plasma ghrelin concentrations

		Male				Female				
	All ($n=105$)	All ($n=49$)	KPMC ($n=13$)	KUH ($n=36$)	*P* value*	All ($n=56$)	KPMC ($n=26$)	KUH ($n=30$)	*P* value*	*P* value**
Parameters										
Age (years)	73·4 ± 6·3	75·0 ± 7·0	72·8 ± 4·7	75·8 ± 7·5	0·119	72·0 ± 5·4	69·4 ± 4·7	74·2 ± 5·1	**0·001**	**0·017**
Height (cm)	155·8 ± 8·4	162·1 ± 6·5	163·1 ± 5·1	161·8 ± 6·9	0·488	150·3 ± 5·5	151·3 ± 4·2	149·3 ± 6·4	0·162	**0·001**
Body weight (kg)	55·5 ± 8·5	58·3 ± 9·0	60·4 ± 4·8	57·5 ± 10·0	0·182	53·1 ± 7·2	54·3 ± 7·2	52·1 ± 7·3	0·261	**0·002**
BMI (kg/m^2)	22·9 ± 2·8	22·1 ± 2·6	22·8 ± 1·9	21·9 ± 2·7	0·205	23·5 ± 2·8	23·7 ± 2·9	23·4 ± 2·8	0·671	**0·008**
Acylated ghrelin (fmol/ml)	10·7 ± 10·7	9·3 ± 11·6	6·4 ± 2·8	10·4 ± 13·3	0·099	11·9 ± 9·8	9·8 ± 8·3	13·8 ± 10·8	0·129	0·213
Desacyl ghrelin (fmol/ml)	63·2 ± 44·8	53·3 ± 41·5	65·1 ± 33·6	49·0 ± 43·7	0·183	72·0 ± 46·1	77·3 ± 51·3	67·3 ± 41·4	0·431	**0·031**
A/D ratio (%)	17·1 ± 9·6	18·0 ± 11·0	11·7 ± 6·8	20·3 ± 11·5	**0·003**	16·3 ± 8·2	12·5 ± 5·6	19·6 ± 8·6	**0·001**	0·379
GH (ng/ml)	2·5 ± 3·7	2·5 ± 4·7	3·9 ± 6·7	2·0 ± 3·7	0·349	2·5 ± 2·6	2·8 ± 2·8	2·2 ± 2·4	0·395	0·929
IGF-I (ng/ml)	125·5 ± 36·8	128·5 ± 41·1	134·9 ± 20·8	126·2 ± 46·3	0·370	122·8 ± 32·7	121·7 ± 28·5	123·8 ± 36·5	0·813	0·440
Insulin (μU/ml)	6·1 ± 4·0	5·0 ± 3·1	4·3 ± 1·2	5·2 ± 3·6	0·222	7·2 ± 4·4	6·6 ± 3·2	7·7 ± 5·3	0·319	**0·004**
Glucose (mg/dl)	94·1 ± 9·0	95·0 ± 9·2	92·5 ± 6·4	96·0 ± 9·9	0·156	93·3 ± 8·9	92·4 ± 9·9	94·1 ± 8·0	0·492	0·323
Leptin (ng/ml)	7·9 ± 6·2	4·4 ± 2·3	4·5 ± 1·3	4·3 ± 2·5	0·825	11·0 ± 6·9	10·2 ± 5·6	11·6 ± 8·0	0·440	**0·001**
HOMA-IR	1·4 ± 1·0	1·2 ± 0·8	1·0 ± 0·3	1·2 ± 0·9	0·136	1·7 ± 1·1	1·5 ± 0·7	1·8 ± 1·4	0·223	**0·007**
Systolic BP (mmHg)	140·2 ± 19·6	136·2 ± 19·5	145·8 ± 20·3	132·7 ± 18·3	0·055	143·7 ± 19·1	142·6 ± 20·2	144·6 ± 18·4	0·699	0·050
Diastolic BP (mmHg)	82·4 ± 10·4	81·0 ± 11·0	87·5 ± 10·0	78·7 ± 10·5	**0·014**	83·6 ± 9·8	85·7 ± 11·4	81·7 ± 7·9	0·141	0·215
Sleeping duration (h)***	6·7 ± 1·0	6·8 ± 1·3	6·7 ± 1·1	6·9 ± 1·3	0·594	6·6 ± 0·8	6·8 ± 0·7	6·5 ± 0·8	0·172	0·437

KPMC, Kyoto Preventive Medical Center; KUH, Kyoto University Hospital.
*KPMC vs KUH; **male vs female; ***information of one male subject in KUH is unknown.
Bold values: $P<0·05$.
Système International (SI) units for GH, micrograms per liter (conversion factor, 1·0); for IGF-I to nanomoles per liter (0·131); for glucose, millimoles per liter (0·05551); for insulin, picomoles per liter (6·945); for leptin, nanomoles per liter (0·08).

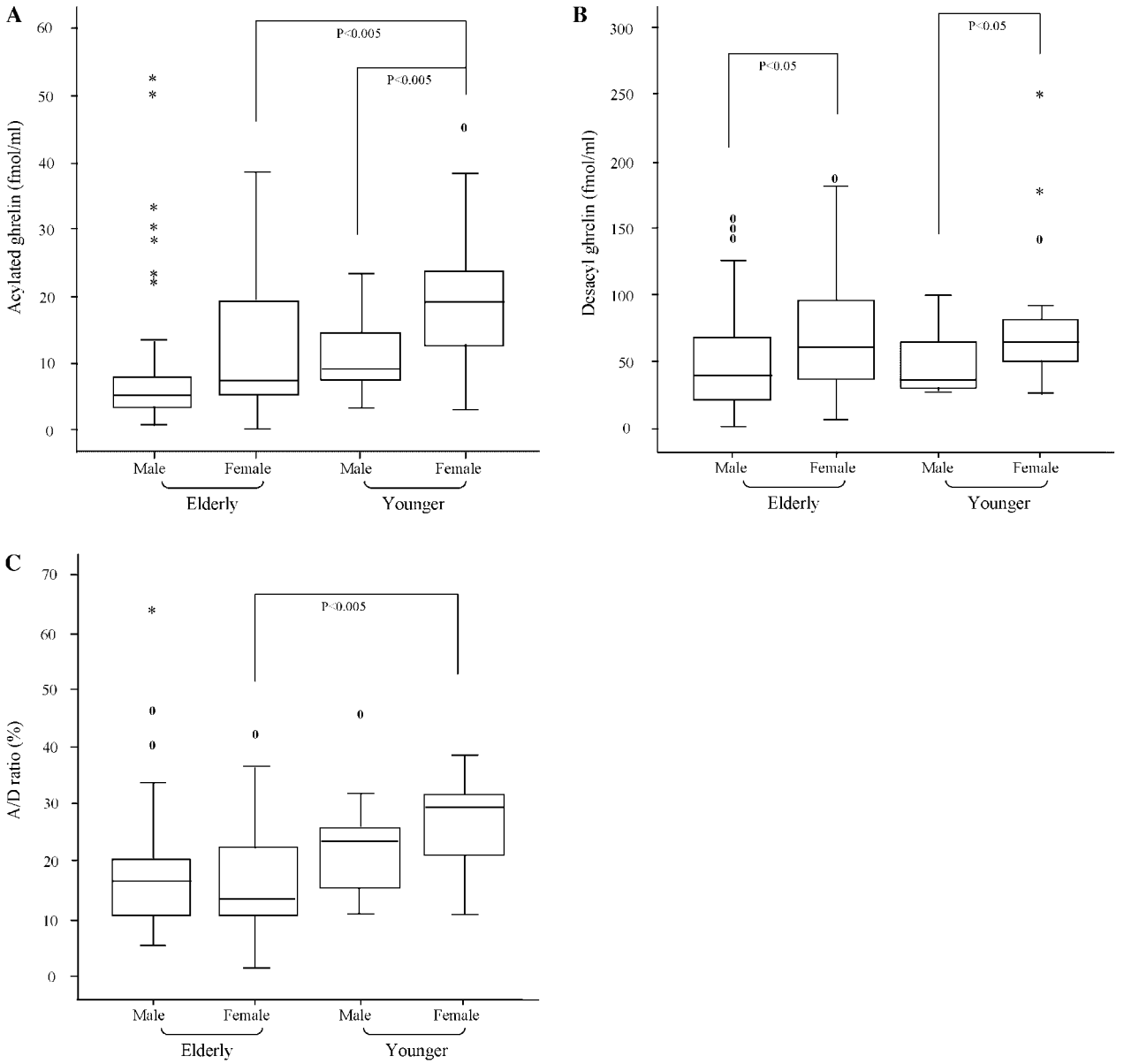

Figure 1 Comparison of plasma acylated ghrelin levels (A), desacyl ghrelin levels (B) and (C) A/D ratios between elderly and younger subjects. The values for subjects younger than 65 years are derived from our previous studies (Akamizu *et al.* 2005). Results are shown as box and whiskers plots. The upper hinge of the box represents the 75th and the lower the 25th percentile. The median is shown as a line across the box. The whiskers above and below the boxes represent the largest and smallest observed scores that are less than 1·5 box lengths from the box. Values farther away are potential outliers. If zero (0) appears, the value is between 1·5 and 3 interquartile ranges from the top or bottom edge of the box. If an asterisk (*) appears, the value is more extreme.

were not significantly different from those in younger female subjects. In addition, plasma levels of desacyl ghrelin in elderly subjects were not associated with gender ($P=0·175$). Although age and blood levels of GH and insulin were correlated with plasma levels of acylated and/or deacyl ghrelin in younger subjects, no parameter correlated with them in elderly subjects or elderly males. In elderly females, however, acylated ghrelin levels positively correlated with IGF-I ($P=0·010$). As this positive correlation between ghrelin and IGF-I levels was surprising, we examined the interaction between BMI and IGF-I by dividing female subjects into two groups based on the median value of BMI, 23·3. Acylated ghrelin levels correlated significantly with IGF-I levels in the group with lower (<23·3) ($P=0·014$), but not higher (>23·3) ($P=0·090$), BMI values (Fig. 2). We did not observe any

Table 2 Mulitiple regression analysis between plasma ghrelin concentrations and various parameters in healthy elderly and younger subjects

	All						**Elderly**											
	Male (n=65)			Female (n=76)			All (n=105)			Male (n=49)			Female (n=56)			**Younger*** (All) (n=36)		
	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)	β	P	r^2 (%)
Acylated ghrelin																		
Site	–	–	–	–	–	–	0·172	0·433	0·7	−0·076	0·831	0·1	0·540	0·087	6·2	–	–	–
Gender	–	–	–	–	–	–	0·366	0·138	2·3	–	–	–	–	–	–	0·196	0·439	2·2
Age group	−0·184	0·661	0·3	0·047	0·914	<0·1	–	–	–	–	–	–	–	–	–	–	–	–
Age	–	–	–	–	–	–	0·001	0·957	<0·1	0·005	0·873	<0·1	−0·012	0·721	0·3	−0·044	**0·002**	31·0
BMI	−0·058	0·333	1·6	0·064	0·281	1·7	−0·026	0·621	0·3	−0·070	0·426	1·7	0·149	0·065	7·2	0·067	0·136	8·1
GH	−0·003	0·906	<0·1	0·046	0·080	4·4	0·015	0·608	0·3	−0·003	0·938	<0·1	0·094	0·123	5·1	0·036	**0·026**	17·1
IGF-I	0·003	0·204	2·8	0·006	**0·011**	9·2	0·006	0·113	2·7	0·003	0·588	0·8	0·015	**0·010**	13·6	−0·001	0·523	1·5
Insulin	−0·032	0·374	1·4	−0·008	0·811	<0·1	−0·015	0·632	0·2	−0·024	0·657	0·5	−0·008	0·847	<0·1	−0·067	**0·015**	20·2
Glucose	−0·001	0·913	<0·1	0·008	0·559	0·5	0·000	0·988	<0·1	0·000	0·993	<0·1	0·008	0·619	0·5	−0·028	0·086	10·6
Leptin	0·015	0·796	0·1	−0·010	0·717	0·2	0·020	0·470	0·6	−0·009	0·920	<0·1	−0·052	0·176	3·9	−0·002	0·904	<0·1
Sleeping time	–	–	–	–	–	–	−0·008	0·938	<0·1	−0·030	0·813	0·1	0·157	0·369	1·8	–	–	–
Desacyl ghrelin																		
Site	–	–	–	–	–	–	−0·317	0·102	2·8	−0·566	0·124	6·1	−0·044	0·858	<0·1	–	–	–
Gender	–	–	–	–	–	–	0·293	0·175	2·0	–	–	–	–	–	–	0·012	0·959	<0·1
Age group	0·076	0·858	<0·1	0·428	0·214	2·3	–	–	–	–	–	–	–	–	–	–	–	–
Age	–	–	–	–	–	–	−0·007	0·670	0·2	−0·004	0·881	<0·1	−0·015	0·550	0·8	−0·014	0·231	5·3
BMI	−0·011	0·848	<0·1	0·022	0·646	0·3	−0·040	0·387	0·8	−0·046	0·604	0·7	0·047	0·457	1·2	0·034	0·409	2·5
GH	0·022	0·447	1·0	0·037	0·075	4·6	0·021	0·407	0·7	0·012	0·756	0·3	0·060	0·213	3·4	0·027	0·063	12·3
IGF-I	0·003	0·264	2·2	0·004	**0·024**	7·3	0·002	0·460	0·6	0·000	0·994	<0·1	0·008	0·094	6·0	0·001	0·467	2·0
Insulin	−0·038	0·297	1·9	−0·023	0·395	1·1	−0·009	0·745	0·1	−0·018	0·740	0·3	−0·007	0·826	0·1	−0·064	**0·013**	20·9
Glucose	−0·010	0·475	0·9	0·010	0·340	1·3	0·006	0·573	0·3	−0·002	0·937	<0·1	0·016	0·212	3·4	−0·024	0·105	9·4
Leptin	0·018	0·766	0·2	0·003	0·903	<0·1	0·025	0·308	1·1	0·020	0·819	0·1	−0·014	0·634	0·5	0·005	0·772	0·3
Sleeping time	–	–	–	–	–	–	−0·036	0·667	0·2	−0·004	0·974	<0·1	−0·002	0·989	<0·1	–	–	–

Bold values: P<0·05.
β: regression coefficient.
r^2 (%): squared partial correlation coefficient.
*The number of younger subjects is too small to be stratified by gender for multiple regression analysis.

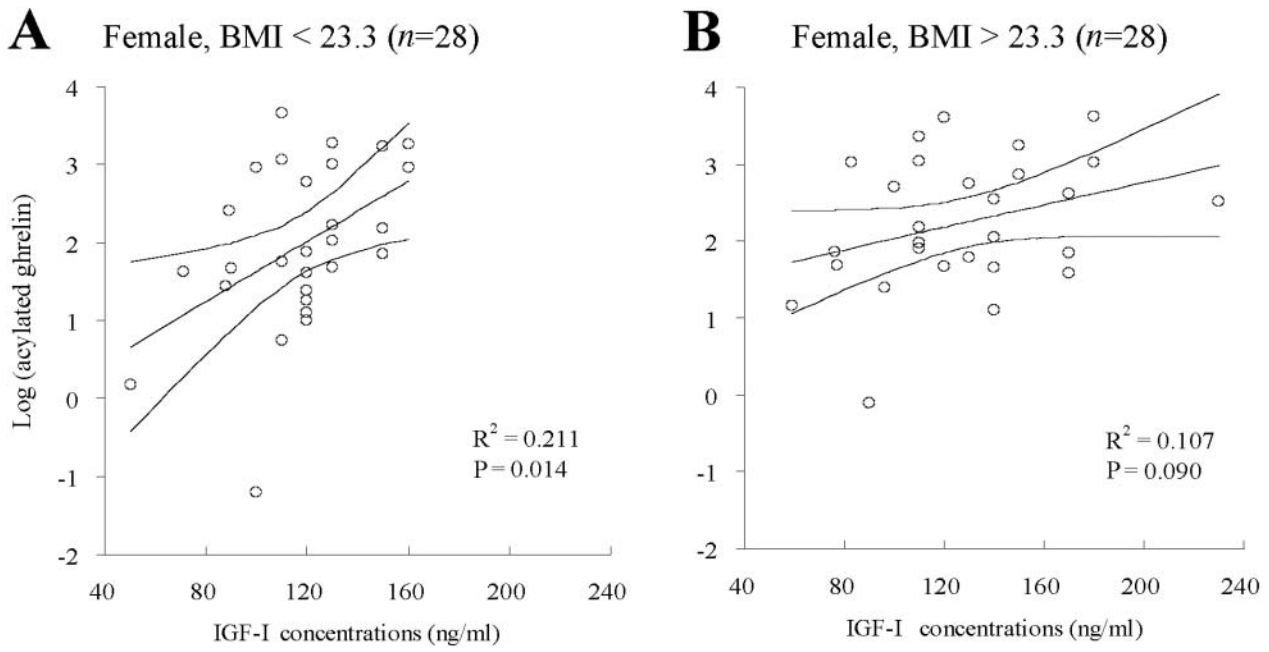

Figure 2 Linear regression analysis of the relationship between IGF-I and plasma acylated ghrelin levels in elderly female subjects of differing BMI levels. (A) Subjects with lower BMI (<23·3, *n*=28); (B) those with higher BMI (>23·3, *n*=28).

significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels in the group with a lower BMI. Although statistically not significant, plasma levels of acylated and desacyl ghrelin in elderly female subjects tended to be positively associated with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3).

In the multivariate model, acylated ghrelin levels in women, but not in men, correlated with systolic BP levels, independently of site, age, BMI, sleeping duration,

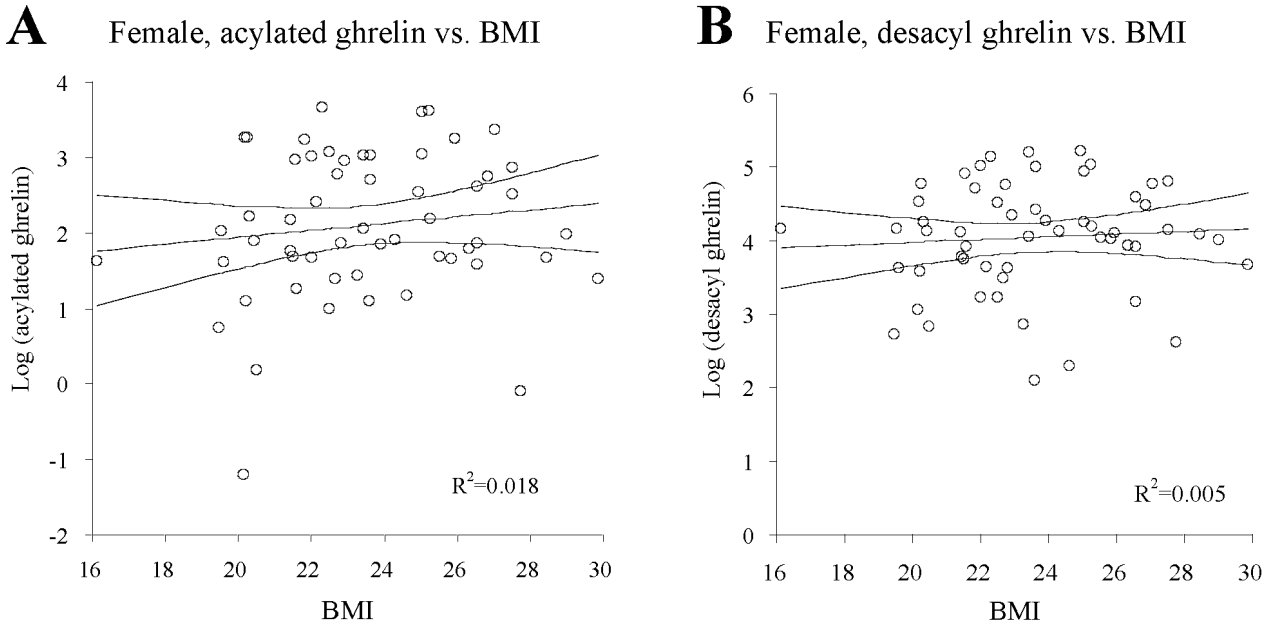

Figure 3 Linear regression analysis of the relationship between BMI and plasma levels of acylated (A) or desacyl (B) ghrelin in elderly female subjects.

Table 3 Relationship between plasma ghrelin concentrations and blood pressure in healthy elderly subjects

	Acylated ghrelin		Desacyl ghrelin		A/D ratio	
	β	*P**	β	*P**	β	*P**
Male						
Systolic blood pressure	−0·003	0·789	0·009	0·467	−0·012	0·050
Diastolic blood pressure	0·012	0·616	−0·016	0·525	0·028	0·027
Female						
Systolic blood pressure	−0·022	**0·039**	−0·016	0·074	−0·006	0·291
Diastolic blood pressure	−0·007	0·719	−0·001	0·969	−0·007	0·565

*Adjusted by recruitment site, age, BMI, sleeping duration, blood pressure (mutually) and blood levels of GH, IGF-I, insulin, glucose and leptin.
Bold values: $P<0·05$.

diastolic BP levels and blood levels of GH, IGF-I, insulin, glucose and leptin ($P=0·039$) (Table 3). Finally, acylated ghrelin levels and A/D ratio in women, but not in men, also correlated significantly with frequencies of bowel movement ($P=0·014$ and $P=0·008$ respectively) (Table 4). In men, desacyl ghrelin levels correlated with this parameter ($P=0·037$). There were no significant correlations between ghrelin levels and smoking habits in any subject groups. Gender difference was not an independent determinant of plasma ghrelin levels; β and *P* values for sex were $\beta=0·366$ and $P=0·138$ (acylated), and $\beta=0·293$ and $P=0·175$ (desacyl) respectively.

Other hormone levels

The correlations between other hormone levels and physiologic parameters in healthy elderly subjects are summarized in Table 5. Significantly, in both sexes, serum GH and IGF-I levels correlated negatively with BMI and age respectively, while serum leptin levels correlated positively with BMI. Plasma glucose levels positively correlated with both serum IGF-I levels and age. Serum IGF-I levels in females correlated positively with plasma concentrations of acylated ghrelin and negatively with serum GH levels, while serum leptin levels in men were significantly associated with age.

Discussion

Although two studies have demonstrated that mean plasma concentrations of total ghrelin in elderly, normal-weight subjects were 36% (Rigamonti *et al*. 2002) and 20% (Sturm *et al*. 2003) lower than those seen in younger, normal-weight subjects, these studies used a small number of subjects. In addition, only total ghrelin levels were examined, and no attempt was made to investigate gender differences. In this study, we demonstrated that the

Table 4 Relationship between plasma ghrelin concentrations and bowel movement in healthy elderly subjects

		Acylated ghrelin		Desacyl ghrelin		A/D ratiio	
	Number	Mean	S.D.	Mean	S.D.	Mean	S.D.
Bowel movement (male)							
≥2/day	15	10·8	10·1	62·9	42·6	15·4	6·0
1/day	29	9·4	13·1	52·7	41·4	17·8	10·3
<1/day	5	4·0	3·1	27·7	34·2	27·0	21·5
P value* (≥1/day vs <1/day)		0·144		**0·037**		0·249	
Bowel movement (female)							
≥2/day	11	15·0	9·8	91·1	43·0	15·6	4·8
1/day	38	12·3	10·2	71·3	47·8	17·3	9·0
<1/day	7	5·5	4·4	45·2	28·6	11·8	6·1
P value* (≥1/day vs <1/day)		**0·014**		0·188		**0·008**	

*Adjusted by recruitment site, age, BMI, sleeping duration and blood levels of GH, IGF-1, insulin, glucose and leptin.
Bold values: $P<0·05$.

Table 5 Mulitiple regression analysis between other hormone levels and various parameters in healthy elderly subjects*

	Male			**Female**		
	Parameters	β	*P*	Parameters	β	*P*
Hormones						
GH						
	BMI	−0·971	0·001	BMI	−0·411	0·001
	Sleeping time	1·347	0·007			
	Leptin	0·610	0·048			
IGF-I						
	Age	−3·262	0·001	Leptin	3·177	0·001
	Glucose	1·540	0·003	Age	−2·317	0·002
	Leptin	4·466	0·028	BMI	−5·987	0·004
				Acylated ghrelin	0·900	0·012
				GH	−3·739	0·013
Insulin						
	BMI	0·602	0·001	Leptin	0·438	0·001
Glucose						
	IGF-I	0·098	0·009	IGF-I	0·128	0·002
	Age	0·528	0·012	Age	0·552	0·040
	BMI	1·006	0·048			
Leptin						
	BMI	0·513	0·001	BMI	1·410	0·001
	Age	0·106	0·017	Insulin	0·506	0·001
				IGF-I	0·044	0·010

β: regression coefficient.
*Multiple regression analysis with backward-elimination procedure was performed after adjustment for the effect of recruitment site; candidate independent parameters were age, BMI, sleeping duration and blood levels of GH, IGF-I, insulin, glucose and leptin.

plasma levels of acylated ghrelin in elderly male and female subjects were respectively 20% and 40% lower than those seen in younger subjects. In contrast, plasma concentrations of desacyl ghelin in elderly subjects of both sexes did not differ from those observed in younger subjects in both sexes. As a result, the A/D ratios in elderly female subjects were significantly lower than those in younger female subjects. In addition, plasma acylated ghrelin levels did not show significant gender difference, while plasma desacyl ghrelin levels in elderly female subjects were significantly higher than those in elderly male subjects, although gender difference was not an independent determinant for them. The reductions in acylated ghrelin levels observed in elderly female subjects may be partially related to a higher BMI than that seen in younger women ($23{\cdot}5 \pm 2{\cdot}9$ vs $20{\cdot}3 \pm 1{\cdot}9$; $P<0{\cdot}001$), as plasma levels of acylated ghrelin in all females were not correlated with age group in the multiple regression analyses. Other modifying factors, especially menopause, should be considered as possibly affecting plasma acylated ghrelin levels in women. In support of this hypothesis, Kellokoski *et al.* (2005) recently reported that estrogen replacement therapy increases plasma levels of acylated ghrelin. Further studies will be necessary to delineate the mechanisms by which estrogen affects the production and/or secretion of acylated ghrelin.

ELISAs used for the measurement of plasma ghrelin levels in this study were two-site sandwich assays with two monoclonal antibodies. One monoclonal antibody recognizes the octanoyl-modified (Active Ghrelin kit) and the other the nonmodified N-terminal portion of ghrelin (Desacyl-Ghrelin kit) (Akamizu *et al.* 2005). The ratio of acylated to (acylated plus desacyl) ghrelin (A/(A+D) ratio) determined by ELISAs was lower than that of acylated to total ghrelin previously determined by RIA, which measures total ghrelin with an antiserum against the C-terminal region of ghrelin. This finding suggests that a fragmented form of ghrelin lacking the N-terminal region may naturally exist in human plasma or may be artificially produced during the RIA procedure. If so, then approximately 40–60% of the total ghrelin measured by RIA is probably fragmented. As a fragmented form of ghrelin is not measured in these two assays, its existence and physiologic implications should be considered and investigated in the future. A limitation of the study was that the measurements of ghrelin concentrations were not undertaken by inclusion of samples from both young and elderly subjects in the same assays. This increases the risk that interassay variation or drift may have reduced our ability to compare concentrations between the two age groups. To mitigate this risk, we used the same kind of assay kit.

A negative correlation between BMI and plasma levels of total or acylated ghrelin was reported by many investigators, including us (Ariyasu *et al.* 2001, Tschop *et al.* 2001, Akamizu *et al.* 2005). On the contrary, plasma levels of both acylated and desacyl ghrelin in elderly female subjects tended to be associated positively with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3). Particularly, the relationship between plasma acylated ghrelin levels and BMI should be noted, although statistically not significant ($\beta=0{\cdot}149$, $P=0{\cdot}065$). These findings suggest that the regulation of ghrelin secretion and/or production in elderly female subjects is altered in comparison to that seen in younger subjects. This altered regulation might be related to the anorexia and undernutrition associated with aging. For example, plasma ghrelin levels may not rise sufficiently when elderly subjects lose weight, resulting in poor appetite and a state of negative energy balance.

In women, acylated ghrelin concentrations correlated positively with IGF-I independently of recruitment site, age, BMI, sleeping duration or blood levels of GH, IGF-I, insulin, glucose and leptin. While a negative correlation between ghrelin and IGF-I levels was reported in children and adolescents (Bellone *et al.* 2002, Whatmore *et al.* 2003), such a correlation has not been observed in adult subjects (Dall *et al.* 2002, Malik *et al.* 2004). Recently, Poykkyo *et al.* (2005) reported a negative correlation between plasma ghrelin and IGF-I in adult subjects with obesity, insulin resistance and type 2 diabetes. The association was particularly strong in both men and subjects in the higher BMI tertiles (maximum: 29·2 or less). In women, the correlation disappeared in the lowest BMI tertile (minimum: 26·5 or more). In agreement with this report, we did not observe a significant correlation between plasma ghrelin and IGF-I levels in the higher BMI population (>23·3). Acylated ghrelin levels, however, correlated positively with IGF-I levels with lower BMI values (<23·3). The positive correlation of ghrelin and IGF-I observed in elderly subjects implicates the dysregulation of ghrelin secretion and/or production during aging, suggesting that the negative feedback regulation of IGF-I may be lost. The IGF-I levels observed in the lower BMI group, $117{\cdot}4 \pm 25{\cdot}1$ ng/ml, may be too low to inhibit ghrelin secretion. In this group, the positive correlation suggests that ghrelin regulates IGF-I production by affecting GH secretion. Although we could not identify significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels, such associations between plasma ghrelin levels and serum GH levels have been observed in previous studies (Yoshimoto *et al.* 2002, Akamizu *et al.* 2005). Thus, the regulation of ghrelin/GH/IGF-I axis in elderly women with low IGF-I levels may be different from that seen in the younger subjects with normal IGF-I levels.

Acylated ghrelin levels in elderly women correlated negatively with systolic BP. The inverse relationship between total ghrelin levels and BP has previously been reported in pregnant women (Makino *et al.* 2002) and patients with hypertension (Poykko *et al.* 2003). Ghrelin, which exerts vasorelaxant or vasodilatory effects *in vitro* (Okumura *et al.* 2002, Shimizu *et al.* 2003), decreases BP (Nagaya *et al.* 2001). Our study also demonstrated the novel correlation of ghrelin levels with frequency of bowel movement in elderly subjects. It should be noted that the smaller number of men than women might have resulted in the borderline correlation between male acylated ghrelin level and the frequency of bowel movement. Ghrelin administration in humans stimulates peristalsis (Takaya *et al.* 2000, Nagaya *et al.* 2001, Akamizu *et al.* 2004) and enhances gastric and intestinal motilities in rats (Masuda *et al.* 2000, Trudel *et al.* 2002, Fujino *et al.* 2003). These findings suggest that ghrelin might play a role in the regulation of bowel motility.

In this study, we confirmed that both blood IGF-I and glucose levels were significantly correlated with age (Davidson 1979, Corpas *et al.* 1993, Muller *et al.* 1999). Serum leptin levels, adjusted for various parameters including BMI, exhibited a significant positive association with age in men (Table 5), but a nonsignificant negative association with age in women (data not shown). This finding corresponds to a report by Baumgartner *et al.* (1999) suggesting that the differences among men and the changes with age in serum leptin levels are associated with differing circulating levels of testosterone. Although plasma glucose levels correlate positively with serum IGF-I levels, few investigators, as far as we know, have reported this positive correlation. As several regulatory factors affect both blood glucose and IGF-I levels, further investigations will be necessary to clarify the mechanisms underlying this correlation. Finally, we confirmed previously observed correlations between BMI and serum GH or leptin levels in elderly subjects (Baumgartner *et al.* 1999, Iranmanesh *et al.* 1991, Chapman 2004).

In summary, we measured plasma levels of acylated and desacyl ghrelin in healthy elderly subjects. The levels of acylated ghrelin in women correlated positively with IGF-I levels, suggesting that the negative feedback mechanism does not function properly in nonobese elderly subjects. These results suggest, however, that ghrelin may regulate IGF-I levels through control of GH. Acylated ghrelin concentrations in women correlated with both systolic BP and the frequency of bowel movements. These findings strongly suggest that, in elderly women, acylated ghrelin may play a role in the regulation of the GH/IGF-I axis, BP and bowel movements. The obvious next step is to explore and confirm these physiologic effects of ghrelin experimentally. In addition, analysis of 24-h acylated and desacyl ghrelin secretion is extremely important to determine the physiologic control of ghrelin secretion during the lifespan. Finally, understanding the relationship between plasma ghrelin levels and these clinical parameters in the elderly may provide therapeutic opportunities to target ghrelin in disorders related to aging.

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