RAPID COMMUNICATION

Plasma levels of active form of ghrelin during oral glucose tolerance test in patients with anorexia nervosa

Yoshikatsu Nakai, Hiroshi Hosoda 1,2, Kazuko Nin 3, Chizuru Ooya, Hiromi Hayashi, Takashi Akamizu 1 and Kenji Kangawa 1,2

College of Medical Technology, Kyoto University, 1 Translational Research Center, Kyoto University Hospital, 53 Kawaharacho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan, 2 Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan and 3 Faculty of Nursing, Shiga University of Medical Science, Shiga, Japan

(Correspondence should be addressed to Y Nakai, College of Medical Technology, Kyoto University, 53 Kawaharacho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan; Email: YN@itan.kyoto-u.ac.jp)

Abstract

Objective: Ghrelin is an acylated peptide, whose octanoyl modification is essential for its biological activities. Previous studies demonstrated that fasting plasma ghrelin levels were high in anorectic patients, suggesting ghrelin may play an important role in the pathophysiology of anorexia nervosa. However, antibodies used in previous work to measure ghrelin concentrations in human blood do not distinguish between the active form of ghrelin (active ghrelin) and desacyl ghrelin with no biological activities. Therefore, we studied plasma levels of active ghrelin during oral glucose tolerance test (OGTT) in anorectic patients, using a radioimmunoassay (RIA) specific for active ghrelin.

Methods: Active ghrelin response to OGTT was evaluated in five female anorectic patients and seven age-matched control females. All subjects were given a 75 g/225 ml glucose solution orally after over-night fasting. For RIA of active ghrelin, 1 N hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma.

Results: Plasma basal levels of active ghrelin were significantly higher in anorectic patients than in controls (52.1 ± 10.5 vs 21.2 ± 3.1 fmol/ml, P < 0.01). They were significantly decreased during OGTT in anorectic patients and in controls, reaching a nadir of 49.0 ± 7.7% and 57.3 ± 4.5% of the basal levels, respectively.

Conclusion: These results suggest that hyperghrelinemia in anorectic patients is caused at least partly by increased secretion of active ghrelin and that glucose ingestion suppresses active ghrelin release in these patients.

European Journal of Endocrinology 149 R1–R3

Introduction

Ghrelin is a 28 amino-residue peptide produced predo- minantly by the stomach with substantially lower amounts deriving from other central and peripheral tissues (1). Ghrelin is a natural ligand of the growth hormone (GH) secretagogue receptor and its acylation in serine 3 is essential for its potent GH-releasing activity. Ghrelin also shows other central and peripheral actions including orexigenic, gastro-entero-pancreatic and cardiovascular activities (2).

In humans, circulating ghrelin levels increased by fasting and decreased by refeeding (3) are reduced in obesity (4, 5) and elevated in anorexia nervosa (4–7), while being negatively correlated to body mass index (BMI) (4–7). It has been hypothesized that ghrelin plays a major role in the endocrine and metabolic response to starvation (2, 8).

The mechanisms controlling ghrelin secretion during fasting and postprandial suppression are unknown. Ghrelin levels were found to be reciprocal to those of glucose and insulin (3). Glucose or insulin might therefore regulate ghrelin release (3, 5, 9). However, there have been contrary reports that plasma ghrelin concentrations are not regulated by glucose or insulin (10).

Anorexia nervosa is a syndrome characterized by abnormal eating behavior and obsessive ideation about body weight in young women (11). The metabolic abnormalities of patients with anorexia nervosa are those of severe malnutrition (11, 12). Active anorectic patients had high plasma ghrelin levels (4–7). However, antibodies used in previous works to measure ghrelin concentrations in human blood do not distinguish between ghrelin and desacyl ghrelin (3–7, 9, 10). The latter has almost no biological activities (1, 13).

In normal subjects, plasma ghrelin levels showed a significant decrease during oral glucose tolerance test (OGTT) (5). However, to our knowledge, there has been no report about the changes of plasma ghrelin levels during OGTT in these patients. Therefore, we studied plasma levels of the active form of ghrelin (active ghrelin) during OGTT in anorectic patients,
using a radioimmunoassay (RIA) specific for active ghrelin (13, 14).

**Subjects and methods**

**Subjects**

The study subjects were five female patients with anorexia nervosa (restricting-type) and seven control subjects. All the untreated anorectic patients who visited our clinic were used for the study except those who had concurrently bulimia nervosa. A diagnosis of anorexia nervosa was made according to the criteria of DSM-IV (11). Control subjects were age-matched healthy women whose BMI was in the range of 19.0–22.5 kg/m². All anorectic patients were amenorrheic and control subjects were studied in the follicular phase of the menstrual cycle. None of them had any associated illness nor any comorbidity. They were receiving no medications when studied. The BMI was significantly lower in anorectic patients than in normal controls (13.9 ± 1.0 vs 20.4 ± 0.5 kg/m²; P < 0.01). All subjects gave their written informed consent for the study.

**Methods**

All subjects were given a 75 g/225 ml glucose solution orally at 0900 h after overnight fasting. Blood was withdrawn from an indwelling flexible catheter into a syringe at 0, 30, 60 and 120 min during OGTT. Plasma samples were prepared as previously described (1, 13). Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Inc., Kyoto, Japan: 1000 kallikrein inactivator U/ml) and centrifuged at 4°C. Plasma glucose was measured by the glucose oxidase method. Serum nonesterified fatty acid (NEFA) levels were measured using an enzymatic method. Serum insulin was measured by immunoradiometric assay (Dainabot Co., Ltd, Tokyo, Japan). For RIA of active ghrelin (N-RIA), 1 N hydrogen chloride was added to the samples at final concentration of 0.1 N immediately after separation of plasma. Peptide was extracted from plasma by Sep-Pak C18 cartridge (Waters Corp., Milford, MA). Active ghrelin concentrations in peptide samples were measured with N-RIA using polyclonal rabbit antibodies raised against the amino-terminal (amino acid positions 1 to 11 with O- N-octanoylation at Ser 3) of ghrelin. The minimal detection limits of N-RIA were 0.4 fmol/tube. The intra- and interassay coefficients of variation were 3.0% and 6.0%, respectively (13, 14).

All results are expressed as means ± S.E.M. Statistical analysis was performed with Mann–Whitney’s U-test or the repeated measures ANOVA and subsequently with Dunnett’s test. P < 0.05 was considered statistically significant. All calculations were performed with programs from SPSS (User’s guide, SPSS 10.0 J for Windows 1999, Chicago, IL).

**Results**

Figure 1 shows the levels of plasma glucose, serum insulin, serum NEFA and plasma active ghrelin during OGTT in anorectic patients and in normal controls. No significant difference was observed in mean basal levels of plasma glucose, serum NEFA and serum insulin between the two groups. Mean basal levels of plasma glucose and serum insulin increased significantly at 30, 60 and 120 min during OGTT in normal controls, and increased significantly only at 120 min during OGTT in anorectic patients. Mean basal levels of serum NEFA decreased significantly at 30, 60 and 120 min during OGTT in normal controls, and decreased significantly at 60 and 120 min during OGTT in anorectic patients (Fig. 1).

Mean basal levels of plasma active ghrelin were significantly higher in anorectic patients than in normal controls (52.1 ± 10.5 vs 21.2 ± 3.1 fmol/ml; P < 0.01). Mean basal levels of plasma active ghrelin significantly decreased at 30 and 60 min during OGTT in normal controls, reaching a nadir of 57.3 ± 4.5% of the basal levels. They also significantly decreased at 60 and 120 min during OGTT in anorectic patients, reaching a nadir of 49.0 ± 7.7% of the basal levels (Fig. 1).

**Discussion**

This study provides the first evidence, as far as we are aware, that high plasma levels of active ghrelin are present in anorectic patients and that they decreased significantly by glucose ingestion in these patients as
well as in normal controls. The levels, measured by N-RIA in the present study, represent those of active form of ghrelin because N-RIA recognizes only the octanoyl-modified portion of ghrelin, which is essential for the biological activity (1, 13). It has been reported that active ghrelin is too unstable to be measured in stored plasma (4). Therefore, 1 N hydrogen chloride was added to plasma samples in the present study since acidification of plasma prevented rapid desacylation of ghrelin (14). Furthermore, peptide was extracted from plasma by Sep-Pak C18 cartridge within 2 months after sampling (13). The mean basal levels of plasma active ghrelin for healthy women in the present study were 21.2 ± 3.1 fmol/ml, which were comparable with those for patients with normal renal function of 14.7 ± 5.8 fmol/ml in the previous study (14).

Anorectic patients had high plasma ghrelin levels in the previous studies with RIAs for C-terminal portion of ghrelin (C-RIAs), which measure desacyl ghrelin as well as ghrelin (4–7). Therefore, elevation in plasma ghrelin levels with C-RIAs could be caused by increased ghrelin secretion, decreased clearance, or by a combination of both factors. In the present study with N-RIA anorectic patients showed 2-fold higher basal levels of plasma active ghrelin compared with normal controls. Furthermore, plasma active ghrelin levels significantly decreased not only in normal controls but also in anorectic patients, suggesting the normal clearance of ghrelin in anorectic patients (Fig. 1). These findings suggest that hyperghrelinemia in anorectic patients is at least partly caused by increased secretion of active ghrelin. Increased bioavailable ghrelin secretion in anorectic patients might reflect a physiological effort to compensate lack of nutritional intake and stored energy (2, 8).

While this manuscript has been prepared for publication, Nedvidkova et al. reported that the acute plasma ghrelin response to food intake is impaired in women with anorexia nervosa (15). The reason of the discrepancies between our results and theirs is not known at present. The possible explanations may be the difference of the assay system (N-RIA vs C-RIA) and the difference of food load (glucose ingestion vs standard meal).

In conclusion, the present findings suggest that hyperghrelinemia in anorectic patients is caused at least partly by increased secretion of active ghrelin and that glucose ingestion suppresses active ghrelin release in anorectic patients as well as in normal controls.

Acknowledgements

This study was supported by a research grant from the Japanese Ministry of Health, Labor and Welfare. The secretarial help of Miss Hitomi Kawamura is greatly appreciated.

References