Plasma Ghrelin Concentrations in Different Clinical Stages of Diabetic Complications and Glycemic Control in Japanese Diabetics

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Abstract. Ghrelin is an acylated 28-amino-acid peptide that stimulates food intake, GH secretion, and gastric motility. Experimental studies have suggested that ghrelin plays roles in glucose homeostasis, atherosclerosis, and microangiopathy. We investigated possible involvement of ghrelin in micro- and macro-vascular diabetic complications and glycemic control in diabetic patients. Fasting and postprandial plasma ghrelin concentrations after a test meal were measured in 108 and 61 Japanese diabetic patients, respectively. Plasma ghrelin concentrations were negatively correlated with body mass index (BMI) ($r = -0.309, P = 0.002$) or HbA$_1c$ ($r = -0.264, P = 0.0065$). Plasma ghrelin levels in patients with diabetic nephropathy who showed high serum creatinine levels (s-Cre) were significantly higher than those in patients who showed normal s-Cre ($P<0.02$). In patients with diabetic triopathy, plasma ghrelin concentrations were significantly lower than those in patients without diabetic complications ($P<0.05$). Stepwise multiple regression analyses revealed that s-Cre, BMI, and HbA$_1c$ were independently associated with plasma ghrelin levels. A postprandial decrease of ghrelin was observed in patients with normal CV$_R$R values or those with normal body weight, whereas it was not seen in obese patients or in patients with low CV$_R$R values. Suppression rates of ghrelin 30–60 min after a test meal in obese patients were significantly lower than those in normal-weight patients. These findings suggest that ghrelin secretion is suppressed by long-term hyperglycemia and that obesity influences the regulation of ghrelin secretion.

Key words: Diabetes, Diabetic complication, Ghrelin, HbA$_1c$, Hyperglycemia

GHRELIN, a GH-releasing peptide isolated from human and rat stomachs, is an acylated 28-amino-acid peptide that stimulates food intake, GH secretion, and gastric motility in many species, including humans [1–4]. Ghrelin is considered to be involved in the regulation of eating behavior and energy homeostasis. For example, chronic ghrelin administration increased the body fat content of rodents [5]. Ghrelin is mainly secreted from endocrine cells in the stomach mucosa, but is also widely expressed in various tissues and cells, including pancreatic β cells [6].

Plasma ghrelin concentrations are negatively correlated with body mass index (BMI) in healthy subjects [7–9] and type 2 diabetic patients without diabetic complications [10]. Low plasma ghrelin concentrations are associated with insulin resistance in patients with polycystic ovary syndrome [11] and in obese children and adolescents [12]. On the other hand, high plasma ghrelin concentrations have been reported in patients who are emaciated due to cancer [13], chronic heart failure [14], or anorexia nervosa [15].

Ghrelin secretion is upregulated under negative energy balance conditions, whereas it is downregulated in a state of positive energy balance. Plasma ghrelin levels were shown to rise before meals and rapidly
decrease after food consumption, glucose intake, or intravenous glucose infusion in humans and rodents [6, 10, 15]. These results indicate that ghrelin secretion is suppressed, at least, under short-term hyperglycemic and/or hyperinsulinemic conditions. The relationship between ghrelin and long-term hyperglycemia, however, has yet to be clarified. In addition, there is data suggesting that ghrelin may have a favorable effect on endothelial function. The ghrelin receptor was up-regulated in the coronary arteries of humans with atherosclerosis [16] and ghrelin was shown to improve endothelial dysfunction through a GH-independent mechanism in rats [17]. Ghrelin also inhibited the angiogenic activity of rat brain microvascular endothelial cells [18]. These findings suggest that ghrelin has an important role in endothelial function and may prevent diabetic complications such as atherosclerosis and microangiopathy.

In the present study, we investigated the relationship between plasma ghrelin concentrations in diabetic patients and micro- and macro-vascular diabetic complications as well as the relationship between plasma ghrelin concentrations and glycemic control.

### Materials and Methods

#### Subjects

One hundred and eight Japanese patients (56 men and 52 women) with diabetes mellitus (11 type 1 and 97 type 2 patients) based on the criteria of the American Diabetes Association [19] were enrolled in our study. The subjects were randomly selected from out- and in-patients who were treated at the Miyazaki Medical College Hospital between 2000 and 2004. Table 1 shows their clinical characteristics. All patients were classified into lean (BMI<18.5), normal-weight (18.5≤BMI<25), or obese (BMI≥25) according to the criteria of the Japan Society for the Study of Obesity. Fifty-one patients were treated with insulin, 30 patients with oral hypoglycemic agents, and 27 patients with diet alone. All patients were clinically stable at the time of evaluation and had no evidence of gastrointestinal disease or cachectic state such as cancer, thyroid disease, liver disease, or infection. Diabetic retinopathy was diagnosed by ophthalmologists using fundus examinations and photography. All patients were classified into one of three grades: (1) no signs of diabetic retinopathy (NDR); (2) simple diabetic retinopathy (SDR); or (3) proliferative diabetic retinopathy (PDR) including pre-proliferative retinopathy [20]. Diabetic nephropathy was classified based on the criteria of the Japan Diabetes Society. Seventy-one patients with normoalbuminuria were classified into stage 1; 16 patients with microalbuminuria stage 2; 14 patients with overt albuminuria but no elevation of serum creatinine levels (s-Cre) stage 3; and seven patients with elevated s-Cre (>1.4 mg/dl) who were not receiving hemodialysis or continuous ambulatory peritoneal dialysis stage 4. Normoalbuminuria, microalbuminuria, and overt albuminuria were defined as <30 mg/day, 30–300 mg/day, and >300 mg/day, respectively. Diabetic neuropathy was diagnosed in the presence of clinical evidence of peripheral sensorimotor neuropathy plus abnormal nerve conduction in at least two or more peripheral

| Table 1. Clinical characteristics of diabetic patients |
|-----------------|----------------|----------------|----------------|
| Lean            | Normal         | Obese          | Total          |
| n (M/F)         | 8 (4/4)        | 61 (31/30)     | 39 (21/18)     | 108 (56/52) |
| Age (y)         | 57.1 ± 2.3     | 56.6 ± 1.8     | 56.7 ± 2.1     | 56.7 ± 1.3  |
| BMI             | 17.4 ± 0.2     | 22.3 ± 0.2     | 28.1 ± 0.8     | 24.0 ± 0.4  |
| Duration of diabetes (y) | 14.4 ± 2.9 | 10.1 ± 1.1 | 10.1 ± 1.3 | 10.4 ± 0.8 |
| Type of diabetes (1/2) | 0/8          | 10/51          | 1/38           | 11/97       |
| HbA1c (%)       | 7.6 ± 0.7      | 8.2 ± 0.3      | 8.0 ± 0.3      | 8.1 ± 0.2   |
| Treatment (diet/OHA/insulin) | 0/2/6      | 10/18/33       | 17/10/12       | 27/30/51    |
| Retinopathy (none/SDR/PDR) | 0/2/6       | 35/15/11       | 22/9/8         | 57/26/25    |
| Neuropathy (+/-) | 6/2          | 31/30          | 25/14          | 62/46       |
| Nephropathy (+/–/3/4) | 4/2/1/1    | 38/11/7/5      | 29/4/5/1       | 71/17/13/7  |
| Macroangiopathy (+/–) | 1/7          | 10/51          | 6/33           | 17/91       |

Data are expressed as means ± SEM. BMI, body mass index; OHA, oral hypoglycemic agents; SDR, simple diabetic retinopathy; PDR, proliferative diabetic retinopathy.
nerves. Diabetic macroangiopathy was diagnosed based on a positive history of ischemic heart disease, cerebral infarction, or arteriosclerosis obliterans.

Protocol

The Institutional Committee of Miyazaki Medical College approved the protocol, and all subjects gave informed consent before participation. Blood samples were collected from all the patients at 0800 h after they fasted overnight. Sixty-one diabetic patients were given a test meal (30% of their daily energy intake containing 50% carbohydrate, 30% fat, and 20% protein) at 0800 h. Their blood samples were collected at 0, 30, 60, and 120 min after eating a test meal.

Laboratory methods

Plasma ghrelin concentrations were measured using a radioimmunoassay developed by our laboratory, which targeted the C-terminal region of human ghrelin as described [10]. Inter- and intra-assay variations were less than 8% and 6%, respectively. The levels of plasma glucose, serum insulin, serum C-peptide, serum creatinine, urinary albumin, urinary creatinine, and HbA1c were measured using standard laboratory methods.

Coefficient of variation of R-R intervals (CV_{R-R})

CV_{R-R} was calculated using the following formula using an electrocardiogram FDX-4521 (Fukuda Denshi, Tokyo, Japan): CV_{R-R} = (standard deviation/mean value of R-R intervals) × 100 (%). CV_{R-R} was calculated during a 60-sec period when it appeared stable on an electrocardiogram.

Statistical analyses

Statistical differences among more than two groups were evaluated using one-way analysis of variance (ANOVA) and a post hoc Fisher’s test. Values are expressed as means ± SEM. Unpaired Student’s t-test and Wilcoxon’s correction for unequal variances were used for comparisons between two groups. Correction coefficients were calculated by linear regression analysis. Stepwise multiple regression analyses were used to determine the contributing factors to plasma ghrelin level. We used the following independent variables: age, sex, BMI, HbA1c, s-Cre, and fasting glucose. A P value of less than 0.05 was considered statistically significant. Statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Plasma ghrelin concentrations in lean diabetic patients were significantly higher than those in the other two groups, and the plasma ghrelin concentrations in normal-weight patients were significantly higher than those in obese patients (Fig. 1A). As plasma ghrelin concentration was negatively correlated with BMI (Fig. 1B), we examined the relationships between the levels of plasma ghrelin and nephropathy in the lean,
normal-weight, and obese patients separately. Plasma ghrelin concentrations in normal-weight patients with stage 4 nephropathy were significantly higher than those in normal-weight patients with other stages of nephropathy (Fig. 2A). A similar result was seen in obese patients (data not shown). Plasma ghrelin concentration was positively correlated with s-Cre levels in normal-weight patients (Fig. 2B) as well as those in obese patients (data not shown). Plasma ghrelin concentration was negatively correlated with creatinine clearance (Ccr) in both normal-weight patients (Fig. 2C) and obese patients (data not shown). We could not analyze these parameters in the lean group due to the small number of patients.

Next, we examined the relationship between plasma ghrelin concentrations and diabetic complications. We compared all groups except for stage 4 nephropathy patients, because plasma ghrelin concentrations in patients with high s-Cre were significantly higher than those in patients with normal s-Cre. Plasma ghrelin concentrations in normal-weight patients were not affected by the presence of retinopathy, neuropathy, or macroangiopathy (Fig. 3A–C). Similar findings were observed in obese patients (data not shown). Plasma ghrelin concentrations in normal-weight patients with diabetic triopathy (i.e. retinopathy, nephropathy, and neuropathy) were significantly lower than those in the normal-weight patients without complications (Fig. 3D). Plasma ghrelin concentration was negatively correlated with HbA$_1c$ in all subjects (Fig. 4). To further define the relationship between plasma ghrelin level and various parameters in diabetic patients, stepwise multiple regression analysis was employed. In this analysis, plasma ghrelin level was used as a dependent variable and age, sex, BMI, HbA$_1c$, fasting plasma glucose, and s-Cre were used as independent variables in all subjects. When plasma ghrelin level was examined, s-Cre entered the regression first ($r = 0.326$), followed by BMI ($r = 0.420$), and HbA$_1c$ ($r = 0.467$) (Table 2).

Plasma ghrelin concentrations in normal-weight patients (n = 36) significantly decreased at 30, 60, and 120 min after eating a test meal (Fig. 5A). In obese patients (n = 21), however, the level of plasma ghrelin was suppressed only at 120 min after eating a test meal. Suppression rates from the basal value of plasma ghrelin concentration at 30 and 60 min after a test meal in obese patients were significantly lower than those in normal-weight patients (Fig. 5A). Plasma ghrelin concentrations in normal-weight patients with normal CV$_{R-R}$ values ($\geq 2\%$, n = 16) significantly decreased at 30, 60, and 120 min after eating a test meal. In patients with low CV$_{R-R}$ values ($<2\%$, n = 19), however, a significant decrease in the ghrelin concentration was not seen at 30 and 60 min after eating a test meal (Fig. 5B).
There were no significant differences in suppression rates of plasma ghrelin at 30 and 60 min after a test meal between patients with normal CV$_{R-R}$ values and patients with low CV$_{R-R}$ values (Fig. 5B). Postprandial plasma ghrelin concentrations were not different depending on the treatments (i.e., insulin therapy, oral hypoglycemic agents, and diet alone) (data not shown).

**Discussion**

This study shows that the plasma ghrelin concentrations in diabetic patients with long-term poor glycemic
control were lower than those in patients with good glycemic control. In addition, the postprandial decrease in the level of ghrelin was significantly smaller in obese patients.

Yoshimoto et al. reported that the plasma ghrelin concentration in patients with mild to severe chronic renal failure was positively correlated with s-Cre, and negatively correlated with the Ccr [21]. Moreover, the plasma ghrelin concentrations in patients with end-stage renal diseases were reduced by 50% after a single course of hemodialysis [21]. Bilateral nephrectomy markedly increased the plasma ghrelin concentration without a significant increase in the ghrelin mRNA level in the stomach of mice [21]. Therefore, the elevated plasma ghrelin concentration in patients with stage 4 nephropathy is considered to be associated with decreased clearance of ghrelin in the kidney.

No correlation between the levels of ghrelin and HbA_{1c} was reported in type 1 diabetic children [22, 23]. We found low plasma ghrelin concentrations in diabetic patients with high HbA_{1c} levels or with diabetic triopathy, a condition that results in long-term poor glycemic control. This finding suggests that the plasma ghrelin concentration may be suppressed by long-term poor glycemic control. The effect of ghrelin on insulin secretion is controversial. Some studies have reported a stimulatory effect, whereas others reported an inhibitory effect [24–27]. Conversely, the plasma ghrelin concentration was suppressed in a hyperinsulinemic euglycemic clamp study in normal subjects, and intensive insulin therapy in type 1 diabetic patients [28, 29]. In addition, ghrelin administration induced an increase in glucose levels in human [26, 30]. The plasma ghrelin concentration was negatively correlated with the serum insulin level and insulin resistance [11]. The relationships between the levels of ghrelin and the levels of insulin, glucose, or insulin resistance are complex. This study found no significant correlations between the plasma ghrelin concentration and the fasting glucose level, the fasting insulin level except for that in patients receiving insulin therapy, or the fasting C-peptide level, as well as the homeostasis model assessment score (data not shown). Due to the subjects’ various insulin secretion capacities, insulin resistance levels, and different treatments they received, the mechanisms underlying the negative correlation between the HbA_{1c} level and the plasma ghrelin concentration cannot be explained.

In this study, there were no significant differences in the plasma ghrelin concentrations in diabetic patients with and without macroangiopathy and retinopathy. There is data suggesting that ghrelin may have a favorable effect on endothelial function [16–18]. Low plasma ghrelin concentrations in diabetic patients with poor glycemic control may be a risk factor for the progression of micro- and macro-vascular complications.

CV_{R-R} is a simple index of diabetic autonomic neuropathy, in particular of vagal neuropathy, and a CV_{R-R} value of 2% has been recognized as a critical level for vagal neuropathy [31, 32]. The vagal nerve conveys ghrelin-mediated signals for GH secretion and food intake from the stomach to the brain [33]. Circulating ghrelin levels in humans were increased and reduced by cholinergic agonists and antagonists, respectively [34]. A postprandial decrease in the ghrelin level was not found in sheep treated with cholinergic blockers [35]. While there was no significant difference in the
postprandial suppression rates of ghrelin between the patients with normal CV_{R,R} values and those with low CV_{R,R} values, a significant postprandial decrease in ghrelin levels was detected only in patients with normal CV_{R,R} values at 30 and 60 min after a test meal. This finding supports the importance of the vagal nerve in the regulation of ghrelin secretion, which will be proved more clearly if some new, simple, and accurate methods to examine diabetic autonomic neuropathy are developed.

In conclusion, the present study demonstrates that ghrelin secretion may be suppressed by long-term poor glycemic control and that obesity may influence the regulation of ghrelin secretion.

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