CLINICAL STUDY

Decreased circulating levels of active ghrelin are associated with increased oxidative stress in obese subjects

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Abstract

Objective: To investigate the relationship between active ghrelin and oxidative stress in obese subjects. **Design:** We measured the plasma levels of free 8-epi-prostaglandin F_{2α} (8-epi-PGF_{2α}, a reliable and systemic marker of oxidative stress) and the active form of ghrelin in 17 obese and 17 normal subjects. The biologically active forms of ghrelin were measured using a commercially available radio-immunoassay kit and free 8-epi-PGF_{2α} was measured using an enzyme immunoassay kit.

Results: The circulating level of active ghrelin was significantly decreased (20.4 ± 2.6 vs 40.9 ± 3.9 fmol/ml, $P < 0.01$) while that of 8-epi-PGF_{2α} was significantly increased (61.5 ± 9.6 vs 17.3 ± 3.4 pg/ml, $P < 0.01$) in obese subjects compared with normal subjects. The plasma levels of active ghrelin and 8-epi-PGF_{2α} were significantly correlated in obese ($r = -0.507$, $P < 0.05$) and in all ($r = -0.577$, $P < 0.01$) subjects. Multivariate analysis showed that the plasma levels of active ghrelin and 8-epi-PGF_{2α} were significantly and independently correlated in all subjects ($F = 7.888$, $P < 0.01$).

Conclusions: There is an inverse correlation between circulating levels of active ghrelin and oxidative stress in obesity. Low circulating levels of active ghrelin may enhance oxidative stress and the process of atherosclerosis in obese subjects.

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Introduction

Ghrelin is a peptide that has recently been isolated from the stomach (1, 2). It stimulates appetite and food intake (3–7). Peripheral effects of ghrelin have recently been the focus of many investigations. For example, it has been reported that ghrelin is involved in glucose and insulin metabolism and that it ameliorates hemodynamic and metabolic disturbances during heart failure (8–11). Li *et al.* reported a protective role of ghrelin against the development of atherosclerosis by its suppressive effect on the redox-mediated cellular signaling in obesity (8).

Several studies have demonstrated that increased oxidative stress in obese subjects contributes to the development of atherosclerosis (12–15). However, whether ghrelin is associated with oxidative stress in obese subjects has not as yet been reported.

In the present study, we evaluated the relationship between ghrelin and oxidative stress by measuring the plasma levels of the active form of ghrelin and

of free 8-epi-prostaglandin F_{2α} (8-epi-PGF_{2α}) in obese subjects.

Subjects and methods

Subjects

This study comprised 17 subjects with obesity (body mass index (BMI) ≥ 25.0) and 17 age-matched normal (BMI < 25.0) subjects (Table 1). BMI was calculated as the body weight (in kilograms) divided by the square of the height (in meters). None of the subjects was receiving any medication or was under any exercise or dietary therapy before the beginning of this study.

None of the subjects had diabetes mellitus according to the diagnostic criteria of the American Diabetes Association on the 75-g oral glucose tolerance test (Trelan G 75; Shimizu, Shizuoka, Japan) (16). Among obese subjects, 3 had hypertension (blood pressure $\geq 140/90$ mmHg) and 5 had hyperlipidemia

Table 1 Clinical characteristics of obese and normal subjects. Data are expressed as the means $\pm$ S.E.M.

	Normal subjects (n=17)	Obese subjects (n=17)
Age (years)	36.0 $\pm$ 1.9	35.5 $\pm$ 1.8
Sex (M/F)	16/1	16/1
Body weight (kg)	66.3 $\pm$ 1.2	75.7 $\pm$ 4.2*
BMI (kg/m ²)	22.2 $\pm$ 0.6	28.7 $\pm$ 1.2*
Body fat weight (kg)	14.6 $\pm$ 0.7	21.3 $\pm$ 1.5*
Visceral fat area (cm ²)	76.3 $\pm$ 6.3	165.7 $\pm$ 9.9*
Subcutaneous fat area (cm ²)	122.7 $\pm$ 8.9	206.3 $\pm$ 20.8*
Total fat area (cm ²)	224.6 $\pm$ 15.1	372.0 $\pm$ 26.5*
Fasting plasma glucose (mmol/l)	4.8 $\pm$ 0.1	5.0 $\pm$ 0.2
Fasting serum insulin (pmol/l)	26.3 $\pm$ 2.1	44.0 $\pm$ 5.7*
Total cholesterol (mmol/l)	5.0 $\pm$ 0.2	5.2 $\pm$ 0.2
Triglyceride (nmol/l)	1.5 $\pm$ 0.2	1.3 $\pm$ 0.2
HDL cholesterol (mmol/l)	1.2 $\pm$ 0.1	1.1 $\pm$ 0.1
Systolic blood pressure (mmHg)	121.8 $\pm$ 2.9	128.9 $\pm$ 3.4*
Diastolic blood pressure (mmHg)	77.4 $\pm$ 2.0	79.9 $\pm$ 2.1
GIR (μ mol/kg per min)	55.7 $\pm$ 3.2	46.5 $\pm$ 2.6*
Plasma ghrelin (fmol/ml)	40.9 $\pm$ 3.9	20.4 $\pm$ 2.6*
Plasma PGF _{2α} (pg/ml)	17.3 $\pm$ 3.4	61.5 $\pm$ 9.6*

* $P < 0.05$ compared with normal subjects.
GIR, glucose infusion rate.

(total cholesterol $\geq$ 220 mg/dl (5.7 mmol/l) or triglyceride $\geq$ 150 mg/dl (1.7 mmol/l)).

Written informed consent was obtained from all subjects before the beginning of the study.

Materials and methods

Several variables measured in blood samples, body fat weight, body fat areas, insulin sensitivity and blood pressure were evaluated in all subjects. Venous blood was collected in the early morning before breakfast and after overnight bed rest. After centrifugation, plasma and serum samples were separated in small aliquots and then frozen at -70°C until use.

The plasma glucose level was measured by an automated glucose analyzer (GA03U; A&T, Yokohama, Japan); the measurement is based on an immobilized glucose oxidase- O_2 acceleration method. Serum insulin was measured using an immunoradiometric assay kit (Insulin Riabead II kit; Dainabot, Tokyo, Japan). The serum levels of total cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol were measured by enzymatic methods using an autoanalyzer (TBA60M; Toshiba, Tokyo, Japan).

Biologically active forms of ghrelin in plasma samples were measured with a commercially available radioimmunoassay (RIA) kit (LINCO Research, St Charles, MO, USA) (10). The minimum detectable concentration of ghrelin with this assay is 3 fmol/ml and the intra- and inter-assay coefficients of variation are 5.1% and 4.2% respectively. The plasma levels of free 8-epi-PGF_{2 α} were measured using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) (14, 15). The intra- and inter-assay coefficients of variation were 7.5% and 9.2%

respectively. The detection limit of this assay is 1.5 pg/ml. The maximum storage period of the samples used to determine the levels of active ghrelin and free 8-epi-PGF_{2 α} was 4 months.

Body fat area was evaluated by computed tomography (CT) as previously described (17). At 0800 h, after fasting overnight for 11 h, all subjects underwent a single abdominal CT scanning at the umbilical level. Any intraperitoneal region having the same density as the subcutaneous fat layer was defined as visceral fat area; this area was measured by tracing contours on films using a computerized planimetric method.

Body fat weight was measured by bioelectric impedance using a TBF-101 (Tanita, Tokyo, Japan).

Insulin resistance was evaluated by the euglycemic hyperinsulinemic clamp technique using an artificial pancreas (STG-22; Nikkiso, Tokyo, Japan) as described (18–20). Briefly at 0800 h, a priming dose of insulin (Humulin R; Shionogi, Osaka, Japan) was administered during the initial 10 min in a logarithmically decreasing manner to raise serum insulin rapidly to the desired level (1200 pmol/l); this level was then maintained by continuous infusion of insulin at a rate of 13.44 pmol/kg/min for 120 min. The mean insulin level from 90 min to 120 min after starting the clamp study was stable (obese subjects: 1180.2 $\pm$ 45.6 pmol/l, normal subjects: 1170.6 $\pm$ 65.4 pmol/l). Blood glucose was monitored continuously and maintained at the target clamp level (5.24 mmol/l) by infusing 10% glucose. The mean amount of glucose given during the last 30 min was defined as the glucose infusion rate (GIR), and this was taken as the marker of peripheral insulin sensitivity.

In addition, we measured blood pressure in the supine position after a rest of 5 min.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Comparisons between obese and normal subjects were carried out using the Mann–Whitney U test. Correlations were evaluated by univariate and multivariate analyses. All statistical analyses were performed using the StatView 5.0 software program (Abacus Concepts, Berkeley, CA, USA) for the Macintosh. A $P < 0.05$ was taken as statistically significant.

Results

A significant decrease in the plasma levels of active ghrelin ($P < 0.01$) and a significant increase in the plasma levels of 8-epi-PGF_{2 α} ($P < 0.01$) were observed in obese subjects compared with normal subjects (Table 1). Obese subjects showed a significant increase in the serum levels of insulin ($P < 0.02$) and a significantly decreased GIR ($P < 0.05$) compared with normal subjects.

The plasma levels of active ghrelin were significantly correlated with the serum levels of insulin ($r = -0.678$, $P < 0.01$), triglycerides ($r = -0.572$, $P < 0.02$) and GIR ($r = 0.604$, $P < 0.02$) in obese subjects. The circulating levels of active ghrelin were also significantly correlated with BMI ($r = -0.358$, $P < 0.05$), body fat weight ($r = -0.458$, $P < 0.01$), visceral ($r = -0.417$, $P < 0.02$), subcutaneous ($r = -0.495$, $P < 0.01$) and total ($r = -0.516$, $P < 0.01$) fat area, serum levels of insulin ($r = -0.415$, $P < 0.02$) and GIR ($r = 0.587$, $P < 0.05$) in all (obese + normal) subjects (Table 2).

The plasma levels of 8-epi-PGF_{2 α} were significantly correlated with BMI ($r = 0.455$, $P < 0.01$), visceral ($r = 0.538$, $P < 0.01$), subcutaneous ($r = 0.455$, $P < 0.01$) and total ($r = 0.495$, $P < 0.01$) fat area, serum levels of insulin ($r = 0.553$, $P < 0.01$) and GIR ($r = -0.475$, $P < 0.01$) in all subjects (Table 3).

The plasma levels of active ghrelin were significantly correlated with the plasma levels of 8-epi-PGF_{2 α} in

Table 3 Correlations between PGF_{2 α} and several variables in obese and normal subjects.

	<i>r</i>	<i>P</i>
BMI (kg/m ²)	0.455	<0.01
Body fat weight (kg)	0.281	0.11
Visceral fat area (cm ²)	0.538	<0.01
Subcutaneous fat area (cm ²)	0.455	<0.01
Total fat area (cm ²)	0.495	<0.01
Fasting plasma glucose (mmol/l)	-0.120	0.51
Fasting serum insulin (pmol/l)	0.553	<0.01
Total cholesterol (nmol/l)	0.226	0.21
Triglyceride (mmol/l)	-0.083	0.65
HDL cholesterol (mmol/l)	-0.260	0.14
Systolic blood pressure (mmHg)	-0.024	0.90
Diastolic blood pressure (mmHg)	-0.007	0.97
GIR (μ mol/kg per min)	-0.475	<0.01

obese ($r = -0.507$, $P < 0.05$) and in all ($r = -0.577$, $P < 0.01$, Fig. 1) subjects. The plasma levels of ghrelin were significantly associated with the plasma levels of 8-epi-PGF_{2 α} after adjustment for BMI in all subjects ($F = 7.888$, $P < 0.01$).

Discussion

This is the first report that demonstrates the relationship between plasma levels of active ghrelin and oxidative stress in obese subjects.

Since decreased plasma levels of ghrelin and increased oxidative stress have been reported in obese subjects it is not surprising that there is an inverse relationship between them (5, 7, 10, 13–15); however our study showed, for the first time, that they are correlated independently of obesity. Recent studies have demonstrated that ghrelin has inhibitory effects on leptin- and endotoxin-induced proinflammatory cytokine production by human monocytes, T cells and

Table 2 Correlations between active ghrelin and several variables in obese and normal subjects.

	<i>r</i>	<i>P</i>
BMI (kg/m ²)	-0.358	<0.05
Body fat weight (kg)	-0.458	<0.01
Visceral fat area (cm ²)	0.417	<0.02
Subcutaneous fat area (cm ²)	-0.495	<0.01
Total fat area (cm ²)	-0.516	<0.01
Fasting plasma glucose (mmol/l)	-0.210	0.24
Fasting serum insulin (pmol/l)	-0.415	<0.02
Total cholesterol (nmol/l)	-0.281	0.11
Triglyceride (mmol/l)	-0.296	0.09
HDL cholesterol (mmol/l)	0.322	0.07
Systolic blood pressure (mmHg)	-0.094	0.60
Diastolic blood pressure (mmHg)	-0.008	0.96
GIR (μ mol/kg per min)	0.587	<0.05

Figure 1 Correlation between the plasma levels of active ghrelin and 8-epi-PGF_{2 α} in all subjects. A significant negative correlation was observed between the plasma levels of ghrelin and 8-epi-PGF_{2 α} ($r = -0.577$, $P < 0.01$). ○, normal subjects; ●, obese subjects.

endothelial cells (8, 21). Li *et al.* reported that ghrelin has potent anti-inflammatory effects in human endothelial cells, through inhibition of tumor necrosis factor- α -induced nuclear factor- κ B activation (8); these authors also reported that ghrelin may interfere with redox signaling by inhibiting cytokine release from cultured human umbilical vein endothelial cells. Although correlation does not prove causation, our present observations suggest that decreased circulating levels of active ghrelin may enhance oxidative stress in obese subjects. Further studies should be carried out to investigate the effect of ghrelin administration on oxidative stress status in obese subjects. On the other hand, Choi *et al.* reported dissociation between the plasma levels of total ghrelin and adiponectin in elderly Korean women (22). Thus, further studies are needed to clarify the relationship between ghrelin and proinflammatory cytokines.

The mechanism by which active ghrelin decreases in the systemic circulation of obese subjects is still unknown (23). Recently we reported decreased plasma levels of active ghrelin in obese patients with type 2 diabetes mellitus, the levels being almost similar to those observed in obese subjects without diabetes (10). In the present study, the plasma levels of active ghrelin were significantly associated with BMI, body fat weight, visceral, subcutaneous and total fat areas in all subjects. It is possible that obesity, but not visceral adiposity, itself regulates the circulating level of active ghrelin by controlling the secretion or metabolism of the protein so as to avoid further development of obesity.

The present study also showed that decreased plasma levels of active ghrelin are significantly correlated with insulin resistance in obese subjects. Similar findings were observed in patients with type 2 diabetes mellitus and obesity (24, 25). The mechanism is unclear but decreased somatotrophic activity of ghrelin due to its systemic deficiency may cause insulin resistance (26).

Recently, measurement of acylated and desacyl ghrelin has been reported in human plasma (27). The ratios of acylated to desacyl ghrelin and acylated to acylated + desacyl ghrelin were significantly correlated with acylated ghrelin, but not with desacyl ghrelin levels, suggesting a decreased activity of acylation enzyme in hypoghrelinemia (27). Further studies should be carried out to clarify whether acylation enzyme activity is decreased in obese subjects.

In brief, the present study showed, for the first time, that circulating levels of active ghrelin are associated with increased oxidative stress in obese subjects. Although correlation does not prove causation, this observation suggests that decreased circulating levels of active ghrelin may lead to increased oxidative stress and contribute to the development of atherosclerosis in obese subjects.

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