Plasma Ghrelin, Body Fat, Insulin Resistance, and Smoking in Clinically Healthy Men: The Atherosclerosis and Insulin Resistance Study

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The purpose of the study was to examine whether insulin sensitivity was associated with fasting plasma ghrelin concentrations in a population-based sample of 58-year-old clinically healthy Caucasian men. The methods used were dual-energy x-ray absorptiometry (DXA) for measurement of body composition and a conventional euglycemic hyperinsulinemic clamp, measuring glucose infusion rate (GIR) that was adjusted for fat-free mass. Plasma ghrelin was measured by radioimmunoassay. The results showed that ghrelin was not associated with GIR adjusted for fat-free mass or with GIR adjusted for body mass, and body fat, or waist circumference. Plasma ghrelin correlated negatively to body fat (−0.46, $P < .001$) and waist circumference (−0.45, $P < .001$). Ghrelin was also inversely related to systolic and diastolic blood pressure ($r = -.29$ and $r = -0.34$, respectively, $P < .01$) and positively to high-density lipoprotein (HDL) cholesterol (0.33, $P < .01$), and low-density lipoprotein (LDL) particle size (0.34, $P < .001$), but these associations did not remain after adjustment for body fat. Plasma ghrelin was associated with current smoking independent of waist circumference. Among current smokers, circulating plasma concentrations were higher in those who had smoked during the hour preceding the blood sample than those who had smoked 2 to 12 hours ago ($P = .043$). The conclusion is that whole body insulin sensitivity was not associated with plasma ghrelin concentrations. Body fatness was the strongest determinant of circulating ghrelin. It was found that acute smoking may affect ghrelin levels.

GHRELIN, a 28-amino acid peptide, is the endogenous ligand for the growth hormone secretagogue receptor.[1] Ghrelin is secreted mainly by the stomach from a distinct endocrine cell type, but it is also produced in small amounts by pancreas, hypothalamus, and other organs.[1,2] Energy balance is a determinant of plasma ghrelin concentration. Fasting increases plasma ghrelin, which decreases after feeding.[3-5] Intravenous ghrelin infusion has been shown to increase food intake in humans.[6] Anorexia is associated with high and obesity with low plasma ghrelin concentrations.[7-9] A 6-month weight reducing diet was associated with a significant increase in plasma ghrelin profile.[10] In rats, the appetite-stimulating effect of ghrelin seems to be mediated by a hypothalamic neuropeptide Y or AGRP pathway.[11-13]

The stimulus for ghrelin production in the stomach is related to glucose and insulin metabolism. In man, oral and intravenous glucose decreases ghrelin, whereas insulin infusion seems to increase plasma ghrelin.[7,14] In rat, gastric distension by water did not affect ghrelin production.[15] Ghrelin is present in α cells of the rat and human pancreas.[2] The ghrelin producing X cells in the stomach and pancreatic α cells may originate from the same endodermal progenitor cells.[2] Both ghrelin and its receptor are present in the pancreatic islets, and ghrelin may affect islet functions via the systemic circulation or in a paracrine manner.[2] Ghrelin has been reported to inhibit insulin secretion in isolated rat pancreas in situ.[16] It has been questioned whether the insulin-resistant state may affect plasma ghrelin concentrations. In women with polycystic ovary syndrome, plasma ghrelin was highly correlated to an indirect measure of insulin resistance, but not to the degree of obesity.[17]

Accordingly the aim of the present study was to examine whether insulin sensitivity measured by the gold standard clamp technique was associated with plasma ghrelin concentrations in a sample of men obtained from the general population.

MATERIALS AND METHODS

The study was performed by using data from the previously published Atherosclerosis and Insulin Resistance (AIR) study[18,19] and frozen plasma (−80°C) for determination of ghrelin concentrations. As previously described in detail, the inclusion criteria were clinically healthy Swedish 58-year-old men.[18,19] A population-based sample of 852 men, 58-years-old, were divided into quintiles of estimated insulin sensitivity, and a random-sample from each of these quintiles was obtained to recruit men with varying degrees of obesity and insulin sensitivity. This group of 104 men (mean age, 58.2 ± 0.17 years) participated in the study. They underwent a euglycemic hyperinsulinemic clamp with the primary aim to examine the association between insulin sensitivity and atherosclerotic disease, as previously reported.[19] Plasma was available for measurement of ghrelin in 100 men. The characteristics have been described elsewhere.[19] Briefly, body mass index (BMI) was 26.3 ± 4.4, waist-hip-ratio .95 ± 0.07 cm, systolic blood pressure 124 ± 20 mm Hg, diastolic blood pressure 73 ± 12 mm Hg, serum triglycerides 1.59 ± 1.17 mmol/L, high-density lipoprotein (HDL) cholesterol 1.26 mmol/L, and plasma ghrelin 321 ± 221 pg/mL (geometric mean 249, SEM 22). The subjects received both written and oral information before they gave their consent to participate. The study was approved by the Ethics Committee at Sahlgrenska University Hospital.

Measurements

Questionnaires were used to evaluate history of previous and current disease. The total number of years of smoking was multiplied by the number of cigarettes smoked daily. The product was called "cigarette-years." Body weight was measured on a balance scale with the subject

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Table 1. Correlations Between Plasma Ghrelin Concentrations and Various Characteristics of the Subjects (N = 100)

Characteristic	Correlation Coefficient (Spearman)
Body mass index (kg/m^2)	−.46‡
Waist circumference (cm)	−.45‡
Waist-hip-ratio	−.38‡
Body fat (kg)	−.53‡
Systolic blood pressure (mm Hg)	−.29†
Diastolic blood pressure (mm Hg)	−.34†
Serum cholesterol	
Total (mmol/L)	.10
HDL (mmol/L)	.33†
Serum triglycerides (mmol/L)	−.16
LDL peak particle size (nm)	.34†
Blood glucose (mmol/L)	.03
Glucose infusion rate (mg/kg body mass/min)	.26†
Glucose infusion rate (mg/kg fat free mass/min)	.19
Plasma insulin (μIU/mL)	−.48‡
Current smoker (n [%])	.23*
Cigarette years	.08

*$P < .05$, †$P < .01$, ‡$P < .001$.

dressed in underwear. Waist circumference was measured with the subject in supine position and hip circumferences with the subject standing. The blood pressure was measured phonographically (Korotkoff sounds recorded on electrocardiogram [ECG] paper) in the right arm after supine rest. Blood pressure was calculated to the nearest 1 mm Hg, and the mean of 2 recordings was used in the statistical analyses. Whole blood glucose was measured with the glucose oxidase technique. Blood samples were drawn, and serum and plasma were frozen in aliquots at −70°C within 4 hours. A euglycemic hyperinsulinemic clamp examination ad modum de Fronzo was performed, slightly modified, as previously published.[20] During the 2 days preceding the day of the examination, subjects were to avoid unusual physical exercise, alcohol consumption, or any major change in caloric intake. Subjects had to fast and avoid smoking or snuff-taking from midnight the preceding day; subjects were allowed to drink water in the morning on the day of the examination. Before the examination started, a questionnaire was completed to verify that the subject had followed the instructions and that there were no signs of respiratory infection or fever. After a priming dose, the insulin infusion rate was 1 mU/min/kg body weight, continuing for 120 minutes until the end of the examination. During the clamp, the target whole blood glucose concentration was 5 mmol/L and the glucose infusion rate (GIR) was adjusted in connection with each determination of whole blood glucose if necessary. After the clamp examination, fat-free mass and total body fat mass were measured using the dual-energy x-ray absorptiometry (DXA) body composition model (Lunar DPX-L, Madison, WI). Two patients were too heavy to be examined by the DXA equipment. Insulin sensitivity was calculated as the GIR per minute adjusted for body weight and fat-free mass during the final hour of the examination.

Laboratory Procedures

Human plasma ghrelin was measured with a radioimmunoassay kit (Phoenix Pharmaceuticals, Belmont, CA) that uses ^{125}I-labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised against full-length octanoylated human ghrelin. The intra-assay coefficient of variation was 8.1% in serum (n = 7). No significant difference was obtained when plasma was compared with serum $r = .94$, and the coefficient of variation was 15.8% (n = 24).

Plasma insulin was assayed on the Access Immunoassay System (Sanofi Pasteur Diagnostics, Chaska, MN) using a 1-step chemiluminescent immunoenzymatic assay. Cross-reactivity with intact proinsulin is <0.2% at 57.6 μIU/mL, 32,33 proinsulin <1% at 57.6 μIU/mL. Between run coefficient of variations are 6.6% at 4.1 μIU/mL (n = 99), 4.8% at 22.0 μIU/mL (n = 102), and 6.0% at 62.9 μIU/mL, respectively.

Low-density lipoprotein (LDL) particle size was assessed on commercially available nondenaturating 2% to 16% polyacrylamide gradient gels (Alamo, San Antonio, TX) as previously described.[21]

Cholesterol and triglyceride levels were determined by fully enzymatic techniques.[18,22] HDL was determined after precipitation of apolipoprotein (apo) B-containing lipoproteins with manganese chloride and dextran sulfate. LDL cholesterol was calculated as described by Friedewald et al.[23]

Statistics

All statistics were analyzed using SPSS for Windows 10.0 (SPSS, Chicago, IL). The results are presented as mean values, standard deviations (SD), and numbers (%). Skewed variables are presented as mean, geometric mean, and standard error of the mean (SEM) and were log transformed before parametric analyses. Nonparametric Spearman's rank correlations coefficients were calculated. Mann-Whitney *U* test was used for comparing continuous variables. Multiple regression was used in the analyses examining the associations between the studied variables. Two-sided $P < .05$ was considered statistically significant.

RESULTS

As shown in Table 1, plasma ghrelin was inversely associated with all measures indicating obesity and abdominal obesity, blood pressure, and plasma insulin. Ghrelin correlated positively to serum HDL cholesterol, and LDL particle size. Plasma ghrelin concentrations showed statistically significant association with GIR adjusted for body mass, but not for fat-free mass.

In Table 2, the results of 6 multiple regression analyses with

Table 2. Multiple Regression Analysis of Covariates to Plasma Log Ghrelin Concentration (log ghrelin is dependent variable) (N = 100)

Model	X_1	β-Coefficient	X_2	β-Coefficient	X_3	β-Coefficient	R^2
1	GIR_{BM}	−0.19	Body fat	−0.61†	—	—	0.24†
2	Log insulin	−0.16	Body fat	−0.34*	—	—	0.22†
3	GIR_{BM}	−0.17	Waist circumference	−0.51†	—	—	0.17†
4	Body fat	−0.94†	Waist circumference	0.45	—	—	0.28†
5	GIR_{BM}	−0.19	Body fat	−0.59†	Smoking	−0.15	0.25†
6	GIR_{BM}	−0.13	Waist circumference	−0.50†	Smoking	−0.21*	0.20†

Abbreviation: GIR_{BM}, glucose infusion rate, adjusted for body mass.
*$P < .05$, †$P < .001$.

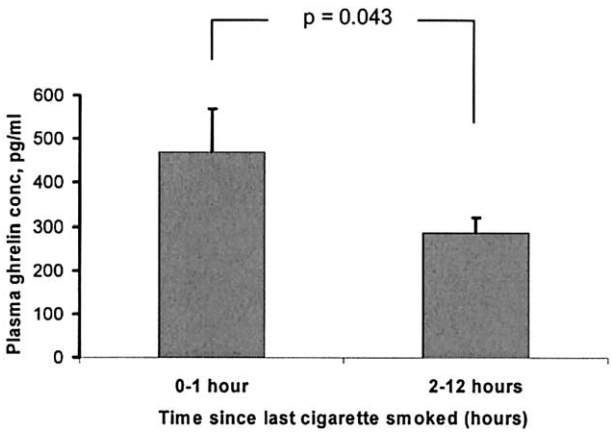

Fig 1. Plasma ghrelin concentration in current smokers (n = 19) divided by the median value (1 hour) of the time since latest cigarette was smoked before the blood sample was taken. Values are geometric mean and SEM.

plasma ghrelin as the dependent variable are presented (model 1 through 6).

Model 4 was the best to explain the variability in plasma ghrelin concentration (R^2=28%) and contained the independent variables, body fat and waist circumference. However, only body fat showed a statistically significant association with plasma ghrelin. Model 1 through 3 showed that GIR adjusted for body mass or log plasma insulin was not associated with plasma ghrelin concentrations independently of body fat or waist circumference. The association between plasma ghrelin and blood pressure and lipid variables did not remain after adjustment for body fat mass (data not shown).

Current smoking status, but not cigarette years, was associated with plasma ghrelin concentrations (Table 1). Those who were current smokers had higher circulating ghrelin levels (geometric mean 348 [SEM 54] pg/mL) than nonsmokers (geometric mean 226 [SEM 23] pg/mL); P = .027). As shown in Table 2, plasma ghrelin was associated with smoking status independent of waist circumference and GIR. After replacing waist circumference with body fat, the independent association between smoking and plasma ghrelin did not remain (Table 2). For the previous smokers, the median time since smoking cessation was 18 years (range, 1 to 38 years). For those who still smoked, there was no statistically significant correlation between the number of cigarettes per day and plasma ghrelin concentration (r = .02). The time interval between the latest smoked cigarette and the time point when the patient came to the laboratory for examination was recorded in 19 smokers (0 to 12 hours; median, 1 hour). As shown in Fig 1, those who had recently smoked had higher plasma ghrelin concentration than those who had abstained from smoking.

DISCUSSION

In this cross-sectional study of 58-year-old men, the association between plasma ghrelin concentrations and insulin sensitivity measured by the current gold standard method was examined. The subjects were obtained from the general population and the participating men were of the same age. This is of importance because circulating ghrelin has been shown to correlate positively with age.[5]

Taken together, the results did not show that insulin sensitivity measured as GIR was related to plasma ghrelin concentrations. First, GIR adjusted for fat-free mass showed no statistically significant correlation to plasma ghrelin. Second, GIR adjusted for body mass showed a statistically significant correlation to plasma ghrelin (r = .26). However, this association did not remain in a multiple regression analysis after adjustment for body fat mass, or waist circumference, as measure of abdominal distribution of fat. This discrepancy relating to the method of normalizing GIR can be explained by differences in the amount of body fat in lean and obese subjects. Adjusting GIR for body mass instead of using fat-free mass or lean body mass leads to a systematic underestimation of insulin sensitivity in obese men.[20] Fasting plasma insulin was not associated with either plasma ghrelin after adjustment for body fat or waist circumference.

There is also indirect evidence from another study that insulin sensitivity is not directly associated with plasma ghrelin concentrations. Shiiya et al[7] studied normal-weight and obese healthy subjects and patients with type 2 diabetes. Type 2 diabetes is almost always associated with insulin resistance,[24] and in that study, there was no difference in plasma ghrelin between normal-weight healthy subjects and normal-weight type 2 diabetics.[7] Also, in obese subjects, there was no difference in ghrelin concentrations between diabetic and nondiabetic groups.[7]

In the previously mentioned study by Shöfl et al,[17] there was a highly statistical association between low plasma ghrelin and insulin resistance. However, the differences between their and our study are many: They examined 26 women of varying ages with polycystic ovary syndrome, and we examined clinically healthy men of the same age; they used indirect measures of insulin sensitivity, such as homeostasis model assessment and continuous infusion of glucose with model assessment, whereas we used the euglycemic hyperinsulinemic clamp method with adjustment for fat-free mass.

The simple correlation analyses showed that ghrelin was related to many factors in the metabolic syndrome apart from plasma insulin concentrations, such as blood pressure, HDL cholesterol, and small LDL particles. However, after adjustment for body fat, these associations did not remain, pointing to the strong relationship between obesity and ghrelin. In accordance with other studies, we observed that measures of body fatness, such as BMI or body fat mass measured by DXA, were strongly associated with plasma ghrelin.[7-9] The same observation was made for such measures of central obesity as waist circumference or waist-hip-ratio. The mechanisms underlying these associations are still unclear. One study found that a weight-reducing diet increased plasma ghrelin levels after 6 months.[10] Another study with both overfeeding, resulting in a weight gain of 8 kg and an energy-reduced diet with a mean weight loss of 5 kg in twins, failed to show any clear change in plasma ghrelin concentrations.[25] In the latter study, the intraclass correlation coefficient was high, indicating that plasma ghrelin is a familial trait.

The limitation of the present study is that only Swedish Caucasian men of one age class were studied. The advantage is

that the variability caused by differences in sex, age, ethnicity, and race could be minimized.

We also observed that current smoking was accompanied by higher plasma ghrelin concentrations than found in nonsmokers. This association remained after adjustment for waist circumference. The impact of cigarette smoking on plasma ghrelin concentration seems to be an acute effect. Thus, those who had smoked during the 60 minutes preceding the blood sample had higher circulating ghrelin levels than those who had not smoked during the last 180 minutes. Cigarette years in current or previous smokers were not related to ghrelin concentration. Neither was there any correlation between the number of daily smoked cigarettes and ghrelin concentration in current smokers. This unexpected observation of an acute effect of smoking is probably a true finding. First, cigarette smoking has a large number of adverse effects on the gastric mucosa, including effects on mucosal blood flow, gastric motility, and levels of free radicals to mention a few.[26] It is also known that tobacco smoking and nicotine are associated with an increase in plasma growth hormone concentration).[27] Second, plasma ghrelin has a fast turnover, increasing sharply before and decreasing rapidly after every meal.[10] Hence, circulating ghrelin concentrations may be more related to the time since latest cigarette smoked than to total consumption over time.

In summary, this is the first study using the euglycemic hyperinsulinemic clamp method examining the relationship between insulin sensitivity and plasma ghrelin concentrations. There was no association between circulating ghrelin levels and insulin sensitivity independent of the degree of obesity. It was also found the plasma ghrelin concentration seems to be acutely effected by cigarette smoking.

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