Ghrelin Improves Endothelial Function in Patients With Metabolic Syndrome

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Background—Metabolic syndrome importantly accelerates the atherosclerotic process, the earliest event of which is endothelial dysfunction. Ghrelin, a gastric peptide with cardiovascular actions, has been shown to inhibit proatherogenic changes in experimental models. This study therefore investigated whether ghrelin administration might beneficially affect endothelial function in metabolic syndrome.

Methods and Results—Endothelium-dependent and -independent vasodilator responses to intra-arterial infusion of increasing doses of acetylcholine and sodium nitroprusside (SNP), respectively, were assessed by strain-gauge plethysmography before and after local administration of human ghrelin (200 μg/min). During saline, the vasodilator response to acetylcholine was significantly blunted (P=0.008) in patients with metabolic syndrome (n=12, 5 female) compared with controls (n=12, 7 female), whereas the vasodilator response to SNP was not different between groups (P=0.68). In patients with metabolic syndrome, basal plasma ghrelin was significantly lower than in controls (P=0.02).

In these patients, ghrelin infusion markedly increased intravascular concentrations of the peptide (P<0.001) and resulted in a potentiation of the vasodilator response to acetylcholine (P=0.001 versus saline) but not to SNP (P=0.22). This effect was likely related to enhanced nitric oxide bioavailability because, in a group of patients with metabolic syndrome (n=6, 2 female), ghrelin had no effect on the vasodilator response to acetylcholine (P=0.78 versus saline) after nitric oxide inhibition by Nω-monomethyl-L-arginine.

Conclusions—These findings indicate that ghrelin reverses endothelial dysfunction in patients with metabolic syndrome by increasing nitric oxide bioactivity, thereby suggesting that decreased circulating levels of the peptide, such as those found in these patients, might play a role in the pathobiology of atherosclerosis. (Circulation. 2005;112:2986-2992.)

Key Words: acetylcholine • endothelium • vasodilation

Metabolic syndrome, the coexistence in the same individual of several risk factors for atherosclerosis, including hyperglycemia, dyslipidemia, and hypertension, is a growing medical problem in Western nations and imposes an unprecedented burden of cardiovascular disease.1 Insulin resistance and obesity are increasingly recognized as the central and causal components of this syndrome,2 even though their mechanistic role in determining vascular damage has not been fully elucidated. Both obesity and insulin resistance have recently been associated with increased production of proinflammatory cytokines,3,4 which might contribute to a state of low-grade, chronic inflammation, as indicated by the elevated plasma levels of acute-phase reactants commonly observed in patients with metabolic syndrome.5 Inflammation, in turn, is known to play a pivotal role in all phases of the atherosclerotic process,6 the earliest event of which is endothelial cell dysfunction.

Ghrelin is a novel growth hormone (GH)–releasing peptide isolated from the stomach, which has been identified as an endogenous ligand for the growth hormone secretagogue receptors (GHS-R).7,8 GHS-R have been detected not only in the hypothalamus and pituitary but also in the cardiovascular system,9 where ghrelin has been shown to exert beneficial hemodynamic effects in both healthy subjects10 and patients with congestive heart failure.11 Of note, recent studies have also reported potent antiinflammatory actions of ghrelin. Thus, both ghrelin and GHS-R are expressed in human T lymphocytes and monocytes, where the peptide acts via GHS-R to specifically inhibit the expression of proinflammatory cytokines, such as interleukin-1β, interleukin-6, and tumor necrosis factor-α.12 More importantly, ghrelin has also been proven able to inhibit proinflammatory cytokine production and mononuclear cell binding in human endothelial cells.13

Owing to these properties, therefore, we hypothesized that ghrelin could exert a protective effect against endothelial dysfunction, especially in conditions such as those clustering in metabolic syndrome, in which circulating levels of the
peptide may be reduced. To this purpose, the present study was designed to assess the effects of the administration of exogenous ghrelin on endothelial function in patients with metabolic syndrome.

Methods

Study Subjects

Eighteen patients (Table) with metabolic syndrome, defined according to the National Cholesterol Education Program’s Adult Treatment Panel III report, were recruited for this study. None of the patients had a history or presence of peripheral vascular disease, coagulopathy, vasculitis, cardiovascular disease, or any other systemic condition. Lipid abnormalities were present in all patients, hypertension in 15 patients, and impaired glucose tolerance in 5 patients; body mass index was 30 in 13 patients and ≥27 in 5 patients. In all cases, waist circumference was >102 cm in men and >88 cm in women (average value = 107 ± 2 cm), thus indicating the presence of central obesity.

Eighteen normal volunteers matched with the patients for sex and approximate age were selected as a control group (Table). Each subject was screened by clinical history, physical examination, ECG, chest x-ray, and routine chemical analyses. None had evidence of present or past hypertension, hyperlipidemia, diabetes, cardiovascular disease, or any other systemic condition.

None of the study participant was taking any medication, including aspirin or vitamin supplements, at the time of the study. In patients with metabolic syndrome taking antihypertensive and/or lipid-lowering drugs, treatment was discontinued for 2 weeks before enrollment into this study. Smokers were asked to refrain from smoking for at least 24 hours before the study; in addition, all participants were asked to refrain from drinking alcohol and beverages containing caffeine for at least 24 hours before the study.

The study protocol was approved by the local institutional review boards, and all participants gave written informed consent.

Protocols

All studies were performed in the morning in a quiet room with a temperature of ~22°C. Each study consisted of an infusion of drugs into the brachial artery and measurement of the response of the forearm vasculature by means of strain-gauge venous occlusion plethysmography. All drugs used in this study were prepared by the local pharmaceutical service following specific procedures to ensure accurate bioavailability and sterility of the solutions.

While the participants were supine, a 20-gauge Teflon catheter (Arrow Inc) was inserted into the brachial artery of the nondominant arm (left in most cases) for drug infusion. Another 20-gauge catheter (Abbott Laboratories) was inserted into a deep antecubital vein of the same arm for blood sampling. This arm was slightly elevated above the level of the right atrium, and a mercury-filled silicone elastomer strain gauge was placed in the widest part of the forearm. The strain gauge was connected to a plethysmograph (model EC-6, D.E. Hokanson Inc), calibrated to measure the percent change in volume, and connected to a personal computer through an analog-to-digital converter. For each measurement, a cuff placed around the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, Hokanson) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressures 1 minute before each measurement to exclude the hand circulation. Flow measurements were recorded for ~7 seconds every 15 seconds; 7 readings were obtained for each mean value. Blood pressure was recorded with the use of a standard mercury manometer. Throughout all studies, volumes infused were matched by administration of variable amounts of saline.

Study 1: Assessment of Vascular Responses to Acetylcholine and Sodium Nitroprusside in Patients and Controls

After the forearm was instrumented, 12 control subjects and 12 patients with metabolic syndrome received intra-arterial infusion of saline for 15 minutes; blood samples were collected, and baseline flow was measured. Forearm blood flow was then measured after the infusion of acetylcholine, an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator. Acetylcholine chloride (Clinalfa AG) was infused at 7, 15, and 30 µg/min, and SNP (Malesci) was infused at 0.8, 1.6, and 3.2 µg/min (the infusion rates were 0.25, 0.5, and 1 mL/min, respectively, for each drug). Each dose was infused for 5 minutes, and forearm blood flow was measured during the last 2 minutes. A 30-minute rest period was allowed, and another basal measurement was obtained between the 2 drug infusions. The sequence of acetylcholine and SNP was randomized to avoid bias related to the order of these procedures.

Study 1: Effects of Ghrelin on Vascular Responses to Acetylcholine and SNP in Patients With Metabolic Syndrome

In the 12 patients with metabolic syndrome participating in study 1, after infusion of acetylcholine and SNP, a 30-minute period of saline infusion was observed to return the forearm blood flow to baseline, and infusion of ghrelin was then started. Human unacetylated ghrelin (Clinalfa) was infused at 200 µg/min (200 µg/mL solution). This dose was selected to achieve an intravascular ghrelin concentration in the infused forearm between 1000 and 5000 pg/mL, which previously has been shown to exert antiatherogenic actions in experimental studies. Ghrelin infusion was then continued for 60 minutes (at 1 mL/min infusion rate), and forearm blood flow was measured every 30 minutes; venous blood samples were again obtained at the end of this infusion period. Then, while ghrelin infusion was maintained unchanged, dose-response curves to acetylcholine and SNP were repeated at the same doses and following the same protocol as before.

Study 2: Effects of Nitric Oxide Inhibition on Vascular Response to Acetylcholine Before and After Ghrelin Administration in Patients With Metabolic Syndrome

To investigate whether the effect of ghrelin on vascular response to acetylcholine in patients with metabolic syndrome might relate to changes in nitric oxide (NO) bioavailability, additional studies were performed with the use of the NO synthase inhibitor Nω-nitro-
were performed by unpaired t test for post hoc comparisons, as appropriate. Group comparisons and 2-way ANOVA for repeated measures followed by Bonferroni t test before.

dose-response curve to acetylcholine was obtained as described unchanged, L-NMMA administration was reinstated, and a fourth 30-minute resting period and while the ghrelin infusion remained acetylcholine was obtained at the end of this period. Finally, after a same time as reported above, and a third dose-response curve to acetylcholine was obtained as described before.

Study 2: Effects of Ghrelin on Vascular Response to Acetylcholine in Control Subjects

To assess the specificity of the effects of ghrelin on vascular response to acetylcholine observed in patients with metabolic syndrome, further studies were performed to investigate the effects of ghrelin administration on vascular response to acetylcholine in control subjects. To this end, 6 new healthy subjects were enrolled and underwent a dose-response curve to acetylcholine during the concurrent infusion of saline, at the same doses and following the same protocol as reported above. After a 30-minute resting period, ghrelin was infused at the same dose and for the same time as reported above, and a third dose-response curve to acetylcholine was obtained as described before.

Biochemical Measurements

Plasma ghrelin was measured in duplicate with the use of a commercial radioimmunoassay kit (Linco Research), which recognizes total (both acylated and unacylated) ghrelin. The intra-assay coefficient of variation in our analysis was 5.2%. Insulin plasma concentrations were determined by electrochemiluminescent immunoassay (Roche Diagnostics). Insulin sensitivity was assessed with the use of the quantitative insulin sensitivity check index (QUICKI = 1/[log(fasting insulin) + log(fasting glucose)]). Serum high-sensitivity C-reactive protein was determined by nephelometry (Dade Behring).

Statistical Analysis

Intragroup analyses were performed by paired t test, 1-way ANOVA, and 2-way ANOVA for repeated measures followed by Bonferroni t test for post hoc comparisons, as appropriate. Group comparisons were performed by unpaired t test and 2-way ANOVA, as appropriate. Correlations were tested by Spearman rank test. All calculated probability values are 2-tailed, and a probability value <0.05 was considered statistically significant. All group data are reported as mean±SEM.

Results

Baseline forearm blood flow was similar in patients with metabolic syndrome and healthy controls (Table). Throughout the studies, mean arterial pressure did not change significantly after infusion of any of the drugs, thus indicating that the drug effects were limited to the infused forearm and did not extend to the systemic circulation.

Plasma Ghrelin Levels in Patients and Controls

Basal plasma ghrelin was significantly higher in control subjects (1155±115 pg/mL) than in patients with metabolic syndrome (837±119 pg/mL;  P<0.02) who participated in study 1. In these patients, ghrelin levels were inversely related to plasma insulin (R=−0.90;  P<0.001) and triglyceride (R=−0.80;  P<0.001) levels and directly related to insulin sensitivity (QUICKI) (R=0.72;  P=0.007); no significant correlation was observed between plasma ghrelin and body mass index, blood pressure, total cholesterol, HDL cholesterol, and glucose (all  P>0.05). In healthy subjects, no significant correlation was found between plasma ghrelin and any of these variables (all  P>0.05).

Vascular Responses to Acetylcholine and SNP in Patients and Controls

During the concurrent administration of saline, infusion of increasing doses of acetylcholine resulted in a progressive increase in forearm blood flow from baseline both in control subjects and in patients with metabolic syndrome participating in study 1. The vasodilator response to acetylcholine, however, was significantly blunted in patients with metabolic syndrome compared with controls (Figure 1, left). Administration of SNP induced a dose-dependent vasodilator response in both normal subjects and patients with metabolic syndrome. In contrast to the acetylcholine results, the vasodilator response to SNP was not significantly different between patients and controls (Figure 1, right).
Effects of Ghrelin on Vascular Responses to Acetylcholine and SNP in Patients With Metabolic Syndrome
Intra-arterial infusion of ghrelin in patients with metabolic syndrome who participated in study 1 resulted in a marked increase in effluent venous levels of the peptide (from 837 ± 119 to 2908 ± 207 pg/mL; \( P < 0.001 \)). Ghrelin administration did not significantly modify basal forearm blood flow (5.2 ± 0.7 mL/min per deciliter at baseline, 5.1 ± 0.6 mL/min per deciliter after 30 minutes, and 5.2 ± 0.8 mL/min per deciliter after 60 minutes of ghrelin infusion; \( P = 0.98 \)). In these patients, however, ghrelin resulted in a significant increase in the vasodilator response to acetylcholine compared with saline (Figure 2, left). In contrast, ghrelin infusion did not result in any significant changes in the vasodilator response to SNP (Figure 2, right).

Effects of NO Inhibition on Vascular Response to Acetylcholine Before and After Ghrelin Administration in Patients With Metabolic Syndrome
Similar to the results obtained in patients with metabolic syndrome participating in study 1, in the absence of NO inhibition by L-NMMA, ghrelin administration resulted in a significant potentiation of the vasodilator response to acetylcholine in patients with metabolic syndrome participating in study 2 (Figure 3, left). In this group of patients, in contrast, the vasodilator response to acetylcholine in the presence of L-NMMA was not significantly modified by ghrelin administration (Figure 3, right).

Effects of Ghrelin on Vascular Response to Acetylcholine in Control Subjects
In control subjects participating in study 2, vascular response to acetylcholine was not significantly different during saline infusion and after ghrelin administration (Figure 4).

Discussion
The main novel finding of this study is that, in patients with metabolic syndrome, ghrelin administration improves the blunted vasodilator responsiveness to acetylcholine; in contrast, vasorelaxation in response to SNP is not significantly modified by ghrelin administration (Figure 4).

Figure 2. Forearm blood flow values in response to intra-arterial infusion of increasing doses of acetylcholine (left) and SNP (right) in patients with metabolic syndrome during infusion of saline or ghrelin (200 \( \mu \)g/min). Values are expressed as mean ± SEM. The probability values refer to the comparison of vascular responses between the treatments by 2-way ANOVA for repeated measures. \(*P < 0.05; \quad **P < 0.01\) at post hoc pairwise comparisons by Bonferroni t test.

Figure 3. Forearm blood flow values in response to intra-arterial infusion of increasing doses of acetylcholine before and after administration of ghrelin at the dose of 200 \( \mu \)g/min in patients with metabolic syndrome in the absence (right) or presence (left) of NO inhibition by L-NMMA. Values are expressed as mean ± SEM. The probability values refer to the comparison of vascular responses between the treatments by 2-way ANOVA for repeated measures. \(*P<0.05; \quad **P<0.01\) at post hoc pairwise comparisons by Bonferroni t test.
Basal plasma ghrelin was significantly lower in our group of patients with obesity-associated metabolic syndrome than in healthy controls. This finding is in agreement with those of previous studies showing reduced circulating ghrelin in patients with obesity. Of note, in obese patients, ghrelin production has been found to be downregulated by obesity-associated insulin resistance rather than by adiposity per se. This notion is strengthened by the results of studies reporting reduced plasma ghrelin in association with other insulin resistance states, such as hypertension, type 2 diabetes, or polycystic ovary syndrome. Taken together, these results suggested that hyperinsulinemia might act as a feedback mechanism to suppress ghrelin production, a concept confirmed by the strong inverse relationship between ghrelin plasma levels and fasting insulin observed in our patients.

Although administration of exogenous ghrelin to patients with metabolic syndrome remarkably increased the peptide concentration in the forearm circulatory bed, it did not result in any change in basal forearm blood flow. This absence of a direct hemodynamic effect of ghrelin is apparently at odds with the results obtained by Wiley and Davenport, who reported that in human mammary artery ghrelin causes vasorelaxation by antagonizing endothelin-1–induced contraction. However, in the study of Wiley and Davenport, vasorelaxation was achieved in vitro by exposing blood vessels to pharmacological concentrations of ghrelin. In our patients, in contrast, the concentrations of ghrelin reached in the local circulation after intra-arterial infusion of the peptide were much lower, being in the high physiological range.

Ghrelin infusion markedly improved endothelium-dependent vasodilation in patients with metabolic syndrome but did not significantly affect the responsiveness to acetylcholine in healthy controls; the latter finding, therefore, enhances the specificity of the effect of ghrelin on endothelial function observed in the metabolic syndrome group. In these patients, the improvement in the vasodilator responsiveness to acetylcholine induced by ghrelin was not observed during NO synthase inhibition with L-NMMA, thereby suggesting that increased availability of NO is the mechanism underlying the beneficial effects of ghrelin on vascular endothelium. The favorable effect of ghrelin on endothelial function seen by us in patients with metabolic syndrome is in agreement with previous observations made in rat aortic rings suspended in bath-organ chambers. Thus, Shimizu et al. reported that repeated subcutaneous administration of ghrelin to GH-deficient rats improves the vasodilator response to acetylcholine through a NO-dependent mechanism. In our study, however, the beneficial effects of ghrelin on endothelial function were already evident 1 hour after the peptide infusion was started. This makes it unlikely that the mechanism underlying the impact of ghrelin on endothelium-dependent vasodilation might relate to increased expression of endothelial NO synthase, as observed by Shimizu et al after long-term administration of the peptide.

Therefore, alternative explanations should be considered for the beneficial effects of ghrelin observed in our study. One putative mechanism might relate to the recently reported antiinflammatory properties of ghrelin. Thus, potent antiinflammatory actions of the peptide have been observed both within the immune system and in human endothelial cells. Other recent observations of significance have linked metabolic syndrome to a state of chronic systemic inflammation, a concept also supported by the increased serum levels of C-reactive protein found in our patients. It is possible to hypothesize, therefore, that ghrelin might act by opposing the deleterious effect of proinflammatory mediators on endothelial cells and then enhance NO bioavailability. Another potential mechanism whereby ghrelin could have favorably affected endothelial function in our patients is by affecting vascular actions of insulin. Previous studies have shown that insulin may physiologically act as an endothelium-dependent vasodilator via activation of endothelial NO synthase through the phosphatidylinositol 3 (PI3)-kinase/Akt pathway; the resultant increase in blood flow has been postulated to contribute significantly to insulin-mediated glucose up-

**Figure 4.** Forearm blood flow values in response to intra-arterial infusion of increasing doses of acetylcholine in normal subjects during infusion of saline or ghrelin (200 μg/min). Values are expressed as mean±SEM. The probability value refers to the comparison of vascular responses between the treatments by 2-way ANOVA for repeated measures.
take.\textsuperscript{25} Conversely, in conditions associated with endothelial dysfunction, interference by insulin-mediated vasoconstriction via activation of the endothelin-1 system\textsuperscript{26} might participate to determine insulin resistance. Recent studies have demonstrated that administration of ghrelin to overweight patients strongly improves their insulin sensitivity.\textsuperscript{27} Moreover, ghrelin has been proven able to exert antiapoptotic effects on endothelial cells through the same PI3-kinase/Akt pathway involved in insulin signaling.\textsuperscript{28} It is possible, therefore, that ghrelin may favorably interact with insulin signaling in endothelial cells, thus restoring the NO-mediated vasodilator effects of the hormone. In this regard, both insulin-sensitizing\textsuperscript{27} and endothelial antiapoptotic\textsuperscript{28} effects of ghrelin are elicited by the unacylated form of the peptide via a receptor subtype likely different from the GHS-R1a mediating the classic neuroendocrine actions of acylated ghrelin.\textsuperscript{29} This consideration, in our view, lends support to the biological significance of the advantageous effects on vascular endothelium observed in our patients after administration of unacylated ghrelin.

It is important to consider that our study was performed in the human intact circulation in vivo; therefore, it is inherently difficult to ascertain the precise mechanism underlying the demonstrated effect of ghrelin on endothelial function in patients with metabolic syndrome. Irrespective of the molecular mechanisms, however, our interventional study with exogenous human ghrelin strongly suggests that the peptide may act as a physiological regulator within the vascular system: a deficiency of ghrelin may be involved in the pathobiology of endothelial damage, whereas increasing its circulating levels may beneficially affect vascular homeostasis. In this regard, lifestyle changes usually recommended to patients with metabolic syndrome as a strategy to reduce their cardiovascular risk burden have also demonstrated the ability to increase circulating ghrelin levels. Thus, diet-induced weight loss in a group of obese subjects has been found to be associated with considerable increase in plasma ghrelin.\textsuperscript{30} In another study, a 1-year exercise program without sedentary, overweight postmenopausal women.\textsuperscript{31} Owing to the beneficial action of ghrelin on vascular endothelium demonstrated in our study, it is possible to speculate that increased circulating ghrelin levels may contribute to the antithromogenic effect of these strategies.

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