Ghrelin Restores the Endothelin 1/Nitric Oxide Balance in Patients With Obesity-Related Metabolic Syndrome

Manfredi Tesauro, Francesca Schinzari, Valentina Rovella, Nicola Di Daniele, Davide Lauro, Nadia Mores, Augusto Veneziani, Carmine Cardillo

Abstract—Obesity is associated with endothelial dysfunction related to decreased NO bioavailability, increased endothelin 1 vasoconstrictor activity, and decreased circulating ghrelin. Therefore, we tested whether exogenous ghrelin may have benefits to improve the balance between endothelin 1 and NO in patients with obesity-related metabolic syndrome. Vasoactive actions of endothelin 1 and NO were assessed in 8 patients with metabolic syndrome and 8 matched controls by evaluating forearm blood flow responses (strain-gauge plethysmography) to intra-arterial infusion of BQ-123 (endothelin A receptor antagonist; 10 nmol/min), followed by N\textsuperscript{\textast}monomethyl-L-arginine (NO synthase inhibitor; 4 \textmu mol/min), before and after infusion of ghrelin (200 ng/min). In the absence of ghrelin, the vasodilator response to BQ-123 was greater in patients than in controls (P<0.001), whereas infusion of N\textsuperscript{\textast}monomethyl-L-arginine induced smaller vasoconstriction in patients than in controls (P=0.006). Importantly, exogenous ghrelin decreased the vasodilator response to BQ-123 (P=0.007 versus saline) and enhanced the magnitude of changes in forearm blood flow induced by N\textsuperscript{\textast}monomethyl-L-arginine (P=0.003) in patients but not in controls (both P>0.05). The favorable effect of ghrelin on endothelin A–dependent vasoconstriction was likely related to the stimulation of NO production, because no change in the vascular effect of BQ-123 was observed after ghrelin (P=0.44) in 5 patients with metabolic syndrome during continuous infusion of the NO donor sodium nitroprusside (0.2 μg/min). In patients with metabolic syndrome, ghrelin has benefits to normalize the balance between vasoconstrictor (endothelin 1) and vasodilating (NO) mediators, thus suggesting that this peptide has important peripheral actions to preserve vascular homeostasis in humans. (Hypertension. 2009;54:995-1000.)

Key Words: abdominal obesity ■ endothelin 1 ■ ghrelin ■ metabolic syndrome ■ NO

Vascular tone is regulated through the actions of locally produced agents and reflects the balance of opposing factors. Among the vasodilators, NO seems to be the most important contributor to the acute regulation of vascular tone, whereas endothelin (ET) 1 is the most potent vasoconstrictor exerting this action principally through type A ET (ETA) receptors.

Endothelial dysfunction in obesity-related metabolic syndrome (MetS) is evident as a failure to vasodilate adequately after exposure to endothelium-dependent vasodilators, but this phenomenon may reflect not only impaired NO bioavailability but also excess vasoconstrictor tone. Findings from both our laboratory\textsuperscript{1} and others\textsuperscript{4} suggest that endogenous ET-1–mediated vasoconstrictor tone is augmented in obesity and may, thus, contribute to vascular damage because of the multiple proatherogenic effects of ET-1.\textsuperscript{5} It is interesting that both ET-1 and NO work as negative feedbacks for each other,\textsuperscript{6} each one acting to limit the action of the other. It is, therefore, possible that ET-1 contributes to endothelial dysfunction both directly, through its vasoconstrictor effects, and indirectly, through inhibition of NO production.

Ghrelin is a newly discovered hormone from the stomach, which induces the release of growth hormone and stimulates food intake, energy balance, and adiposity.\textsuperscript{7-9} Evidence in animal models indicates that ghrelin has a variety of growth hormone-releasing–independent cardiovascular activities, including enhancement of vasodilation and regulation of blood pressure.\textsuperscript{10,11} Of note, low ghrelin concentration has been associated with several features of MetS, including obesity,\textsuperscript{12-14} high blood pressure, and diabetes mellitus.\textsuperscript{15} Most importantly, we have observed previously that local administration of ghrelin ameliorates endothelial dysfunction in patients with MetS.\textsuperscript{16} In vitro studies of human internal mammary arteries from patients with coronary artery disease have shown that ghrelin affects the ET-1 system by counteracting the vasoconstrictor effects of ET-1.\textsuperscript{17} We hypothesized, therefore, that the vascular actions of ghrelin might influence the balance between the ET-1 system and the NO pathway. To explore this hypothesis, we undertook studies with BQ-123, an antagonist of ETA receptors, and N\textsuperscript{\textast}monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase.
Assessment of the Effects of Ghrelin on Vascular Responses to ETA Receptor Blockade and NO Synthase Inhibition

Eight patients with MetS and 8 matched controls underwent assessment of vascular responses to ETA receptor blockade and NO synthase inhibition, both in the absence and in the presence of exogenous ghrelin (study 1, Figure 1). To this purpose, after basal measurements were obtained, infusion of BQ-123 (Clinalfa), a selective antagonist of ETA receptors, was performed at the dose of 10 nmol/min for 60 minutes, and forearm blood flow was measured every 10 minutes. Then, while maintaining constant the administration of BQ-123, infusion of L-NMMA (Clinalfa) was superimposed at 4 μmol/min for 15 minutes, and forearm blood flow was measured again at the end of this period. Afterward, after a 15-minute resting period to allow forearm blood flow return to baseline, acylated ghrelin (Clinalfa) was infused at 200 ng/min for 30 minutes, and forearm blood flow was measured at the end of this time. Then, while maintaining ghrelin infusion unchanged, BQ-123 and L-NMMA infusions were repeated as before.

Methods

Study Subjects

Eighteen patients with obesity-related MetS (Table), defined according to the National Cholesterol Education Program Adult Treatment Panel III report, were recruited for this study. Increased waist circumference was present in all of the patients; lipid abnormalities were present in 12 patients; hypertension was present in 15 patients; and impaired glucose tolerance was present in 7 patients. None of the patients had a history or presence of peripheral vascular disease, coagulopathy, vasculitis, cardiovascular disease, or any other systemic condition. Body mass index was ≥30 kg/m² in 15 patients and ≥27 kg/m² in 3 patients.

Eight (lean body mass index: <27 kg/m², normal waist circumference) volunteers, matched with the patients for age and sex, were selected as a control group (Table). Each subject was screened for clinical history, physical examination, ECG, chest x-ray, and routine chemical analyses. None had evidence of present or past hypertension, hyperlipidemia, diabetes mellitus, cardiovascular disease, or any other systemic condition.

None of the study participants were taking any medication, including aspirin or vitamin supplements, at the time of the study. In patients with MetS taking antihypertensive and/or lipid-lowering drugs, treatment was discontinued for 2 weeks before enrollment into this study. All of the participants were asked to refrain from smoking and drinking alcohol and beverages containing caffeine for ≥24 hours before the study. The local institutional review boards approved the study protocol, and all of the participants gave written informed consent.

Protocols

Each study consisted of infusion of drugs into the brachial artery and measurement of the response of the forearm vasculature by means of strain-gauge venous occlusion plethysmography, according to a methodology reported previously in detail.14 Volumes infused throughout the studies were matched by administration of variable amounts of saline.

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Assessment of the Potential Mechanism of the Vascular Effects of Ghrelin

To ascertain the potential mechanism involved in the effects of ghrelin on ETA-dependent vasconstrictor activity in patients with MetS, 10 additional patients were recruited for 2 different protocols. To verify whether activation of endothelin B (ETB)-mediated vasodilation might contribute to the vascular effects of ghrelin, 5 patients with MetS were studied during nonselective ET(AB) receptor blockade (study 2, Figure 1). To this purpose, patients underwent combined infusion of BQ-123 (10 nmol/min) and BQ-788 (Clinalfa; 5 nmol/min), a selective ETB receptor blocker, with subsequent NO synthase inhibition by L-NMMA, both in the absence and the presence of ghrelin, following an infusion protocol similar to study 1.

On the other hand, to assess whether the inhibitory effect of ghrelin on ETA-mediated vasodilation might relate to inhibition of ET-1 production by increased availability of NO, the effects of ghrelin on the vascular response to BQ-123 were assessed in 5 patients with MetS during administration of exogenous NO (study 3, Figure 1). To this end, continuous infusion of a small dose (0.2 μg/min) of the NO donor sodium nitroprusside (Malesci) was performed, and vascular response to BQ-123, given at the same dose and for the same time as above, was assessed both in the absence and in the presence of ghrelin.

Statistical Analysis

Sample size was calculated a priori for the primary hypothesis that ghrelin might reduce the vasodilator response to BQ-123 in patients with MetS. These calculations showed that a sample size of 8 patients would allow detection of a 30% decrease by ghrelin in forearm blood flow during ETA antagonism, with a power of 80% at α=0.05.

Within-group analyses were performed by paired t test and ANOVA for repeated measures, as appropriate. Group comparisons were performed using unpaired t test and analysis of variance, as appropriate. All of the calculated P values are 2-tailed, and a P<0.05...
was considered to indicate statistical significance. All of the group data are reported as mean±SEM.

Results
Baseline forearm blood flow was similar in patients with MetS and 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.

Vascular Responses to ET_A Receptor Blockade and NO Synthase Inhibition
In control subjects, forearm blood flow values went from 3.4±0.4 mL/min per deciliter at baseline to 3.7±0.6 mL/min per deciliter after 60 minutes of BQ-123 infusion; in patients with MetS, by contrast, infusion of BQ-123 resulted in a marked forearm blood flow increase (from 3.7±0.4 mL/min per deciliter at baseline to 5.9±0.6 mL/min per deciliter after 60 minutes). As a consequence, the degree of vasodilation after blockade of ET_A receptors was significantly higher in patients with MetS than in controls (Figure 2, left), thereby indicating enhanced ET_A-mediated vasoconstrictor tone in these patients.

In control subjects, L-NMMA administration during the concurrent blockade of ET_A receptors was associated with a decrease in forearm blood flow from 3.7±0.6 mL/min per deciliter to 2.5±0.3 mL/min per deciliter; in patients with MetS, however, only a mild forearm blood flow decrease was observed after infusion of L-NMMA (from 5.9±0.6 to 5.2±0.5 mL/min per deciliter). NO synthase inhibition, therefore, resulted in a relative decrease in forearm blood flow (calculated as the percentage of change from values immediately preceding the beginning of L-NMMA infusion) lower in patients than in controls, implying an impaired NO-dependent vasodilator activity in the vessels of these patients (Figure 2, right).

Effects of Ghrelin on Vascular Responses to ET_A Receptor Blockade and NO Synthase Inhibition
Intra-arterial infusion of ghrelin for 30 minutes did not significantly change unstimulated forearm blood flow from baseline in either group (both P>0.05). In patients with MetS, during ghrelin administration, forearm blood flow values went from 3.9±0.5 mL/min per deciliter at baseline to 5.4±0.6 mL/min per deciliter after 60 minutes of BQ-123 infusion. Therefore, the relative forearm blood flow response to BQ-123 in these patients was significantly lower after infusion of ghrelin than before (Figure 3, left), indicating blunted ET_A-dependent vasoconstrictor activity after ghrelin.

During ghrelin infusion, L-NMMA administration during the concurrent blockade of ET_A receptors in patients with MetS was associated with a decrease in forearm blood flow from 5.4±0.6 to 3.2±0.4 mL/min per deciliter. The percentage fall in forearm blood flow induced by NO synthase inhibition during blockade of ET_A receptors, therefore, was significantly higher in the presence than in the absence of ghrelin (Figure 3, right), thereby suggesting an effect of ghrelin to increase NO bioavailability.
In contrast with these results, exogenous ghrelin did not modify the vasodilator response to BQ-123 in control subjects (from 7.8% to 11.6% at 60 minutes; \( P = 0.67 \)); similarly, the magnitude of changes in forearm blood flow induced by \( \mathrm{L-} \)NMMA during ETA receptor antagonism in this group was not modified by ghrelin (28.5% during saline versus 30.5% after ghrelin; \( P = 0.81 \)).

**Effects of Ghrelin on Vascular Responses to Nonselective ETA/B Receptor Blockade and NO Synthase Inhibition in Patients With MetS**

In the absence of ghrelin, the vasodilator response to the combined infusion of BQ-123 and BQ-788 was significantly lower in patients with MetS participating in study 2 (32.7% at 60 minutes) than in those who received BQ-123 alone in study 1 (60.7%; \( P < 0.001 \)), a finding suggestive of preserved ETB-mediated vasodilator capacity in MetS. Administration of ghrelin did not significantly modify the vascular response to nonselective ETA/B receptor antagonism (23.8%; \( P = 0.51 \)) but was associated with enhanced vasoconstrictor effect of NO synthase inhibition by \( \mathrm{L-} \)NMMA (Figure 4), thus implying an effect of the peptide to increase NO bioavailability, even in the presence of ETB receptor blockade.

**Effects of Ghrelin on Vascular Response to ETA Receptor Blockade During Administration of Exogenous NO in Patients With MetS**

In patients with MetS participating in study 3, continuous infusion of low doses of sodium nitroprusside resulted in a blunted vasodilator response to ETA receptor antagonism (\( P < 0.001 \)) compared with patients who received BQ-123 alone in study 1, thereby suggesting decreased ETA-dependent vasoconstrictor tone during administration of exogenous NO. Importantly, in the presence of sodium nitroprusside, exogenous ghrelin no longer affected the vasodilator effect of BQ-123 (Figure 5).

**Discussion**

The main finding of the present study is that the enhanced ETA-dependent vasoconstrictor activity present in patients with MetS is reduced by exogenous ghrelin. Furthermore, ghrelin infusion is able to reverse the impaired NO-dependent vasodilator tone in these patients. Taken together, these findings suggest that, in addition to its central action to regulate food intake, ghrelin may have important vascular effects to restore the physiological balance between vasoconstrictor and vasodilator forces, thus importantly contributing to the maintenance of vascular homeostasis in humans.

In the absence of ghrelin, ETA antagonism by BQ-123 resulted in a greater vasodilator effect in patients than in controls, thereby suggesting that ET-1–mediated vasoconstrictor tone is indeed augmented in patients with MetS. In addition, NO synthase inhibition by \( \mathrm{L-} \)NMMA during blockade of ETA receptors was associated with lower vasodilator response in patients than in controls, a finding suggestive of reduced NO bioavailability in the arteries of these patients. In contrast with the results observed in patients, ghrelin infusion did not affect the vascular response to selective ETA antagonism in healthy subjects; similarly, the vasodilator response to NO synthase inhibition by \( \mathrm{L-} \)NMMA in our control group was not different before and after ghrelin administration. Taken in conjunction, these findings enhance the specificity of the vascular effects of ghrelin to normalize the altered ET-1/NO balance within the vasculature of patients with MetS and suggest that the favorable actions of exogenous ghrelin on vascular homeostasis become apparent in disease states, such as obesity, where endothelial dysfunction is associated with diminished circulating ghrelin. Although plasma levels of ghrelin before and after its infusion were not measured in the current study, our previous work using the same methodology has shown clearly that exogenous ghrelin is able to restore normal plasma concentrations of the peptide in patients with obesity-related MetS, in whom ghrelin circulating levels were decreased. Alternatively, because ghrelin is expressed in endothelial cells of human arteries, where it plays a functional role in the autocrine/paracrine regulation of vascular tone, exogenous ghrelin could replace...
impaired local synthesis of the peptide by dysfunctional endothelial cells of patients with MetS.

Mechanisms of the Vascular Effects of Ghrelin

Different mechanisms might be evoked to explain the favorable effect of ghrelin on the vascular balance between ET-1 and NO observed in our patients. The blunted vasodilator response to selective ETA blockade observed after ghrelin administration suggests an effect of the infused peptide to either reduce the sensitivity of vascular smooth muscle cells to the contractile effects of ET-1 or to decrease the production of ET-1 by endothelial cells. The former hypothesis lends support from previous ex vivo studies of human internal mammary arteries, showing that preincubation with ghrelin blunts the vasoconstrictor response to ET-1.17 This mechanism alone, however, is unable to explain the enhanced bioavailability of NO during ETA antagonism, as indicated in our patients by the increased magnitude of the response to 1-NNMMA observed after ghrelin infusion, hence the necessity to consider other possibilities.

Previous experimental studies have clearly demonstrated the ability of ghrelin to downregulate ET-1 production. Thus, ghrelin improves tissue perfusion in rats subjected to sepsis by affecting prepro–ET-1 gene expression and inhibits tumor necrosis factor-α–induced ET-1 secretion in endothelial cells by interfering with the nuclear factor κB pathway.20 In addition, exogenous ghrelin attenuates the progression of chronic hypoxia-induced pulmonary hypertension in rats by decreasing the overexpression of ET-1 mRNA.21 All of these effects of ghrelin, however, are exerted through actions at the gene expression level, a mechanism that requires a longer time course than the ghrelin infusion period used in our protocol, which makes these mechanisms unfit to explain our findings.

An alternative hypothesis relies on previous evidence that the vasodilator response to selective ETA blockade in the forearm circulation of healthy subjects involves ET-1 stimulation of endothelial ETB receptors,22 with an ensuing increase in NO production; NO, in turn, may suppress ET-1 production,23 thereby contributing to reduced ETA-dependent vasoconstriction. In addition, studies in insulin-resistant states have shown restoration of NO bioavailability after ETA receptor blockade in obese patients,24 suggesting the possibility that ET-1, present at an increased concentration in the arterial wall of these patients and blocked from acting via ETA receptors, may then exert enhanced action via ETB receptors. The possible involvement of ETB-mediated, NO-dependent vasodilation in the vascular effects of ghrelin was investigated in our study by use of nonselective ETA/ETB blockade. If this hypothesis were correct, one would have expected no changes in NO-mediated vasodilator tone after ghrelin administration during dual ETA/ETB antagonism. In our study, however, ghrelin administration resulted in enhanced magnitude in the response to 1-NNMMA, even in the presence of blocked ETB receptors, which makes the ETB hypothesis unlikely to explain our results.

Another option to be considered involves a direct effect of ghrelin to enhance NO bioactivity as the possible mechanism leading to the suppression of ET-1 production. This possibility was tested in a group of patients by evaluating the effects of ghrelin on ETA-mediated vascular tone during constant infusion of an exogenous NO donor. Of note, ghrelin administration under those conditions did not affect ETA-dependent vasoconstrictor tone; this implies that, in the presence of adequate NO concentrations within blood vessels, there is no additive effect of ghrelin and that increased NO bioavailability is the likely mechanism underlying the observed inhibitory effect of ghrelin on the ET-1 system.

Inflammation and increased oxidative stress have been involved in the pathophysiology of endothelial dysfunction and vascular damage in conditions associated with insulin resistance and hypertension.25,26 It might, therefore, be postulated that ghrelin acts to enhance NO bioavailability by decreasing oxidative stress, given its ability to downregulate the expression of inflammatory cytokines.27 An alternative way by which ghrelin may affect NO is by stimulating its production. Of note, ghrelin administration during dual ETA/B antagonism. In our study, however, expands those previous observations and furthers our knowledge into the mechanisms involved in the effects of ghrelin in the human circulation. Thus, because vascular physiology is a complex balance of opposing forces, inhibition of ET-1 activity within blood vessels importantly adds to the vasculoprotective properties of ghrelin.

Conclusions

Previous work has led to the discovery that the vasoactive effects of ghrelin are not limited to the acylated form of the peptide, the only ligand for the GHS-R1a receptor mediating its central orexigenic actions and growth hormone release,7 but are shared by deacylated ghrelin.19 The latter form does not interact with the orexigenic pathways28 and is, hence, devoid of those untoward effects, like increased appetite and weight gain. All of these considerations lead us to believe that the relevance of our current findings may extend beyond their pathophysiological interest and acquire clinical significance.

Perspectives

MetS is associated with increased cardiovascular morbidity and mortality. Better understanding of the underlying mechanism for these detrimental effects, therefore, is crucial in trying to reduce the significant burden of the disease. In this study, we demonstrated that exogenous ghrelin is able to reduce ET-1–dependent vasoconstriction and to improve NO bioavailability in the vessels of patients with MetS. The likely mechanism underlying these effects of ghrelin is increased NO bioactivity, leading, in turn, to inhibition of ET-1 production. On the basis of these findings, it is interesting to speculate whether the ghrelin system could not only provide a link between obesity and its major cardiovascular conse-
quences, such as hypertension and atherosclerosis, but also be the target of novel strategies for cardiovascular prevention.

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Disclosures

None.

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