Vascular Effects of Obestatin in Lean and Obese Subjects

Francesca Schinzari,[1] Augusto Veneziani,[2] Nadia Mores,[3] Angela Barini,[4] Nicola Di Daniele,[5] Carmine Cardillo,[1] and Manfredi Tesauro[5]

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Obese patients have impaired vasodilator reactivity and increased endothelin 1 (ET-1)–mediated vasoconstriction, two abnormalities contributing to vascular dysfunction. Obestatin, a product of the ghrelin gene, in addition to favorable effects on glucose and lipid metabolism, has shown nitric oxide (NO)–dependent vasodilator properties in experimental models. Given these premises, we compared the effects of exogenous obestatin on forearm flow in lean and obese subjects and assessed its influence on ET-1–dependent vasoconstrictor tone in obesity. In both lean and obese participants, infusion of escalating doses of obestatin resulted in a progressive increase in blood flow from baseline (both $P < 0.001$). This vasodilation was predominantly mediated by enhanced NO activity, because N^G-monomethyl-L-arginine markedly blunted the flow response to obestatin in both groups (both $P < 0.05$ vs. saline). In obese subjects, antagonism of ET_A receptors by BQ-123 increased forearm flow during saline ($P < 0.001$) but did not induce additional vasodilation ($P > 0.05$) during obestatin. Circulating obestatin levels were not different between lean and obese participants ($P = 0.41$). Our findings indicate that obestatin causes NO-dependent vasodilation in the human circulation. This effect is preserved in obesity, where it is accompanied by reduced ET-1–mediated vasoconstriction. These latter observations make obestatin a promising target for vascular prevention in obesity and diabetes.

OBESITY STUDIES

According to the current figures of the World Health Organization, the worldwide prevalence of obesity is still on the rise, carrying an increased burden of type 2 diabetes and other untoward consequences, especially cardiovascular complications. Impaired vasodilator reactivity has been recognized as an early hemodynamic abnormality characteristic of these patients (1), but also increased vasoconstrictor tone, predominantly resulting from enhanced endothelin (ET)-1 activity (2,3), has been shown to importantly contribute to their vascular dysfunction and damage.

Obestatin was identified in 2005 as a ghrelin-associated peptide derived from alternative splicing of the common precursor prepro-ghrelin and was originally reported to reduce food intake and gastric emptying through activation of the G-protein–coupled receptor (GPCR) GPR39 (4). Even though these effects on feeding behavior and gastrointestinal motion have been subsequently disputed and the precise identity of its cognate receptor(s) is still a matter of debate (5), obestatin indisputably exerts a variety of effects in different cell types, including pancreatic β-cells, where it increases survival and proliferation by inhibiting apoptosis and inflammation (6,7). In line with these actions, other favorable effects of obestatin have been observed on glucose and lipid metabolism, such as increased glucose uptake and insulin sensitivity as well as inhibition of lipolysis in human adipocytes (7,8).

Interestingly, in addition to its helpful metabolic properties, obestatin has been shown to provide vascular benefits in experimental models. Thus, in rat aorta and the superior mesenteric artery, Agnew et al. (9) have demonstrated that obestatin favorably affects endothelial function, inducing nitric oxide (NO)–dependent relaxation via an adenylate cyclase–linked GPCR. These findings have been more recently confirmed in the mouse cerebral artery, where obestatin induces NO-dependent vasodilation, which is maintained during ghrelin receptor antagonism (10). This effect is also present in animals with increased superoxide generation caused by ghrelin receptor knockout, hence suggesting an additional mechanism for the vascular protection afforded by obestatin (10).

[1]Department of Internal Medicine, Catholic University, Rome, Italy
[2]Department of Surgery, Catholic University, Rome, Italy
[3]Department of Pharmacology, Catholic University, Rome, Italy
[4]Department of Biochemistry, Catholic University, Rome, Italy
[5]Department of Internal Medicine, University of Rome Tor Vergata, Rome, Italy

Corresponding author: Carmine Cardillo, carmine.cardillo@unicatt.it.

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Owing to the advantageous metabolic and vascular actions observed in preclinical models, we hypothesized that obestatin might become an interesting target for cardiovascular disease prevention in human obesity and diabetes. To this purpose, the current study was designed to compare the effects of obestatin on forearm flow in lean and obese subjects and to investigate whether inhibition of ET-1–dependent vasoconstriction might be an additional mechanism of the vascular action of obestatin in obese individuals.

RESEARCH DESIGN AND METHODS

Study Subjects

The study recruited lean subjects (BMI <25 kg/m^2, normal waist circumference) and individuals with central obesity (waist circumference $\geq$102 cm for men or $\geq$88 cm for women) (Table 1), without or with the metabolic syndrome (defined according to the National Cholesterol Education Program's Adult Treatment Panel III) (11). All participants had no history or current evidence of cardiovascular disease (coronary artery disease, cerebrovascular or peripheral occlusive arterial disease, coagulopathy, vasculitis) or any other systemic condition. In obese participants taking antihypertensive and/or lipid-lowering drugs, treatment was discontinued for at least 1 week before the vascular study. Blood pressure was repeatedly measured during this time, and when needed, treatment was restarted with the exclusion of the subject from the study. No participants smoked, and all were asked to refrain from drinking alcohol and beverages containing caffeine for at least 24 h before the study. The participants were not engaged in programs of regular physical activity. Because of the possible effects of sex hormones on vascular activity of the ET-1 system (12), all female participants were studied within the first week from the beginning of their menstrual cycle. The study protocol was approved by the institutional review boards of the University of Rome Tor Vergata and Catholic University, and all participants gave written informed consent before their participation in the study.

Protocols of Vascular Reactivity Studies

All studies were performed in a quiet room with a temperature of ~22°C (Supplementary Figs. 1 and 2). Each study consisted of infusions of drugs into the brachial artery and measurement of forearm blood flow 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. Participants were asked to fast for at least 8 h before the study. While participants were supine, a 20-gauge Teflon catheter (Arrow Inc., Limerick, PA) was inserted into the brachial artery of the nondominant arm (left in most cases) for drug infusion. Another 20-gauge catheter (Abbott Laboratories, Abbott Park, IL) was inserted into a deep antecubital vein of the same arm for blood sampling.

The extended arm was positioned slightly above the level of the right atrium and a Hg-filled strain gauge was placed around the widest part of the forearm. The strain gauge was connected to a plethysmograph (model EC-6; Hokanson Inc., Bellevue, WA) calibrated to measure the percentage 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 mmHg with a rapid cuff inflator (model E-10; Hokanson Inc.) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressures 1 min

Table 1—Clinical characteristics of the study population

		Obese subjects		
	Lean subjects (n = 14)	No metabolic syndrome (n = 13)	Metabolic syndrome (n = 11)	P value
Sex				
Male	7	5	6	
Female	7	8	5	
Age, years	40 ± 3	38 ± 3	41 ± 3	0.74
BMI, kg/m^2	23 ± 1	38 ± 2*	42 ± 2*	<0.001
Waist, cm	82 ± 4	122 ± 5*	117 ± 3*	<0.001
Blood pressure, mmHg				
Systolic	116 ± 2	124 ± 3*	133 ± 3*#	0.01
Diastolic	74 ± 4	79 ± 3	84 ± 4	0.17
Glucose, mg/dL	88 ± 3	89 ± 2	95 ± 5	0.30
Cholesterol, mg/dL				
Total	162 ± 8	199 ± 13	191 ± 13	0.07
HDL	49 ± 3	48 ± 3	40 ± 2	0.15
Triglycerides, mg/dL	92 ± 8	117 ± 17	164 ± 26*	0.02
Insulin, μU/mL	8 ± 1	19 ± 3*	18 ± 3*	0.008

Data are expressed as number of subjects or as mean ± SEM. Comparisons were performed by one-way ANOVA. There were no differences between the subgroups of lean or obese subjects participating in the two different protocols. *$P < 0.05$ vs. lean subjects. #$P < 0.05$ vs. no metabolic syndrome at the Holm-Šídák post hoc test for multiple comparisons.

before each measurement to exclude the hand circulation. Flow measurements were recorded for ~7 s every 15 s. Seven readings were obtained for each mean value. Blood pressure was recorded with the use of a standard Hg manometer. Throughout all studies, volumes infused were matched by administration of variable amounts of saline.

Protocol 1: Assessment of the Effects of Obestatin and Acetylcholine on Vascular Tone in Lean and Obese Subjects

To assess the effects of obestatin on forearm flow, 5 lean and 14 obese subjects were enrolled. After the forearm was instrumented, venous samples were drawn to determine basal plasma concentrations of obestatin and ghrelin, normal saline was infused intra-arterially for 15 min, and basal flow was measured. Each participant then received incremental doses of obestatin (Bachem AG, Weil am Rhein, Germany) from 0.2 to 3.2 nmol/min. These doses were selected to achieve intravascular concentrations of the peptide similar to those previously shown to induce relaxation in arterial preparations in vitro (9). Each dose of obestatin was given for 5 min, and venous samples and flow measurements were obtained at the end of each period. A 15-min period to allow flow to return to baseline was observed, blood flow was measured again, and an intra-arterial infusion of acetylcholine, which is known to at least partly induce NO-dependent vasodilation, at the dose of 7.5, 15, and 30 μg/min, was started. Each dose of acetylcholine was given for 5 min, and flow measurements were obtained at the end of each dose. After that, a saline washout of 20 min was observed, and the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 4 μmol/min) was infused for 15 min. Unstimulated flow was reassessed at the end of this period, and the dose-response curves to obestatin and acetylcholine were repeated as before.

Protocol 2: Assessment of the Effects of Obestatin on Vascular Responses to ET_A Receptor Blockade in Obese Subjects

To investigate the possible effect of obestatin to inhibit obesity-related ET-1–dependent vasoconstrictor tone, 10 additional obese subjects were recruited for a study using the selective ET_A receptor antagonist BQ-123. Nine additional age-matched lean subjects were also included in this protocol to collect blood samples for measurement of plasma levels of obestatin; however, they did not undergo vascular reactivity testing with BQ-123 because previous studies in our laboratory have repeatedly demonstrated little contribution of ET-1 to the maintenance of basal vascular tone in lean subjects (13,14). In obese participants, after the forearm was instrumented and saline was given for 15 min, baseline flow was measured; at that point, an infusion of BQ-123 (Bachem) was started at the dose of 10 nmol/min for 60 min, and blood flow was measured every 10 min. After a 20-min resting period to allow flow to return to baseline, infusion of obestatin (0.8 nmol/min) was started for 20 min, and forearm flow was reassessed. Then, while constant obestatin administration was maintained, the BQ-123 infusion was repeated as before.

Analytical Procedures

Plasma levels of obestatin and total ghrelin (acylated and desacylated) were measured by enzyme immunosorbent assay kits (Peninsula Laboratories, San Carlos, CA, and Phoenix GmbH, Karlsruhe, Germany, respectively).

Statistical Analysis

Group comparisons were performed by unpaired *t* test and by one-way and two-way ANOVA, as appropriate. Within-group analyses were performed by paired *t* test and by one-way and two-way ANOVA for repeated measures, as appropriate. The Holm-Šídák test was used for post hoc comparisons when needed. When preliminary testing showed that the data being compared did not have normal distribution and equal variance, nonparametric tests were applied (Mann-Whitney or ANOVA on ranks, as appropriate). The primary hypotheses of the study were that in obese participants, obestatin might increase unstimulated blood flow by 30% and reduce the vasodilator effect of ET_A receptor antagonism by 50%. A priori calculations based on figures of previous studies with similar end points showed that a sample size of 14 participants in protocol 1 and 10 participants in protocol 2 could allow detection of these within-subject effects of obestatin with a power of 80% at $\alpha = 0.05$. All other comparisons were considered secondary end points. All calculated probability values were two-tailed, and a P value of <0.05 was considered statistically significant. All group data are reported as mean ± SEM.

RESULTS

During the performance of the vascular studies, mean arterial pressure and heart rate did not change significantly after infusion of any of the drugs used in the study, thus indicating that the drug effects were limited to the infused forearm and did not extend to the systemic circulation.

Effects of Obestatin and Acetylcholine on Vascular Tone in Lean and Obese Subjects

In the participants in this protocol, infusion of escalating doses of exogenous obestatin during saline resulted in a progressive increase of the effluent venous levels of the peptide, without affecting ghrelin levels (Fig. 1).

During saline, administration of obestatin in lean subjects ($n = 5$) was associated with a significant rise ($P < 0.001$) in forearm flow from baseline (Fig. 2, left panel). Similar results were observed in obese individuals ($n = 14$), with a significant vasodilator response achieved after infusion of obestatin ($P < 0.001$) (Fig. 2, right panel). To account for baseline differences in forearm flow, we also compared group differences in the vasodilator response to obestatin as percentage changes (15). We observed that the increase in blood flow from baseline after administration of obestatin was higher in lean than in obese subjects (Fig. 3, left panel). Among our obese participants, the average obestatin-related increase in forearm flow tended to be higher in those without ($n = 7$; 29 ± 3%)

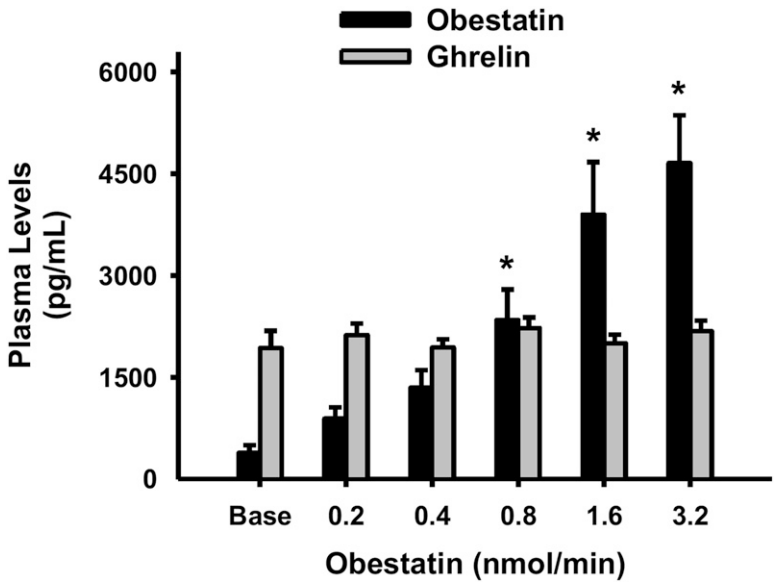

Figure 1—Plots showing effluent venous concentrations of obestatin and ghrelin during intra-arterial infusion of escalating doses of obestatin. All values are means ± SEM. *$P < 0.05$ vs. baseline at one-way ANOVA for repeated measures, followed by the post hoc Holm-Šídák test for multiple comparisons.

than in those with the metabolic syndrome ($n = 7$; 22 ± 3%), but this difference did not reach statistical significance ($P = 0.10$).

Infusion of L-NMMA induced a significant decrease in unstimulated forearm flow in both lean ($P = 0.01$) and obese subjects ($P = 0.006$). During NO synthase inhibition, infusion of obestatin did not result in significant changes of blood flow from baseline in either group (both $P > 0.05$); as a result, forearm flow values during the administration of obestatin were lower during L-NMMA than during saline, both in lean (Fig. 2, left panel) and obese subjects (Fig. 2, right panel). Also, the percentage changes in flow induced by obestatin during L-NMMA were not different between lean and obese participants (Fig. 3, right panel).

During saline, infusion of escalating doses of acetylcholine resulted in a significant increase of forearm flow from baseline both in lean and obese subjects (both $P < 0.001$; Fig. 4). The degree of acetylcholine-related vasodilation, however, was significantly lower in obese than in lean participants ($P = 0.02$).

NO synthase inhibition by L-NMMA blunted the vasodilator effect of acetylcholine both in lean and obese subjects

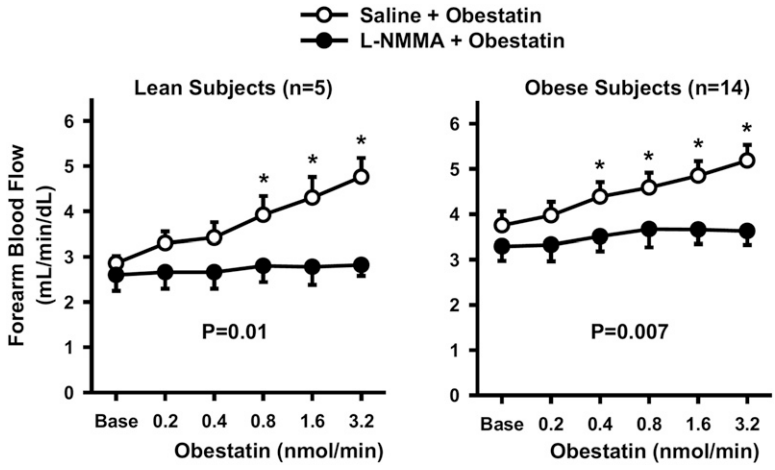

Figure 2—Plots showing blood flow responses to intra-arterial infusion of escalating doses of obestatin during the concomitant infusion of saline or L-NMMA in lean subjects (left panel) and obese subjects (right panel). The *P* values refer to the comparisons of vascular responses to obestatin between saline and L-NMMA by two-way ANOVA for repeated measures. All values are means ± SEM. *$P < 0.05$ vs. baseline at one-way ANOVA for repeated measures, followed by the post hoc Holm-Šídák test for multiple comparisons.

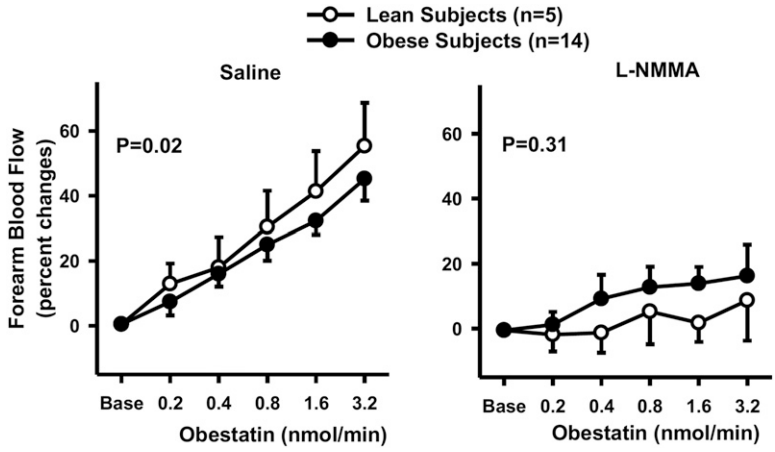

Figure 3—Plots showing the comparison of percentage changes in blood flow from baseline in response to intra-arterial infusion of escalating doses of obestatin between lean subjects and obese subjects, during the concomitant infusion of saline (left panel) or L-NMMA (right panel). All values are means ± SEM. The *P* values refer to the comparisons of vascular responses to obestatin between groups by two-way ANOVA.

(Fig. 4). After L-NMMA administration, the vasodilator response to acetylcholine was no longer different between the two groups ($P = 0.29$).

Effects of Obestatin on Vascular Response to ET_A Receptor Blockade in Obese Participants

In obese participants in protocol 2 ($n = 10$) during saline, ET_A receptor antagonism resulted in a significant increase in forearm flow (Fig. 5*A*, left panel). Administration of obestatin in these individuals resulted in a significant vasodilation; however, BQ-123 did not induce additional changes in flow during the infusion of obestatin (Fig. 5*A*, right panel). To account for the baseline flow imbalance resulting from obestatin-mediated vasodilation, we also compared the vascular responses to ET_A receptor blockade as percentage changes and observed that the increase in flow from baseline was markedly higher during saline than during obestatin (Fig. 5*B*). The average obestatin-related decrease in BQ-123–dependent vasodilation tended to be lower in obese individuals without ($n = 6$; 23 ± 4%) than in those with the metabolic syndrome ($n = 4$; 31 ± 4%); however, this difference did not reach statistical significance ($P = 0.09$).

Plasma Concentrations of Obestatin in Lean and Obese Subjects

Fasting obestatin plasma levels were modestly lower (21%) in obese (398 ± 72 pg/mL) than in lean subjects (504 ± 114 pg/mL), but this difference was not statistically significant ($P = 0.41$).

DISCUSSION

To our knowledge, this is the first study investigating the effects of exogenous obestatin in the intact human circulation in vivo. Its novel findings are that this ghrelin-associated peptide is able to induce vasodilation in the forearm vessels

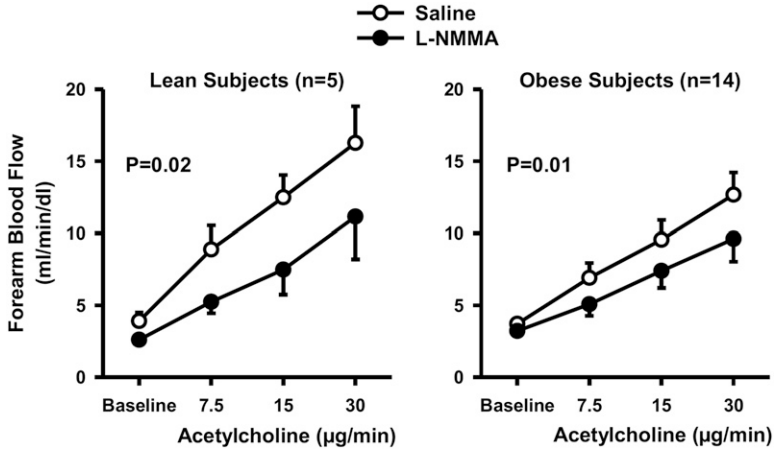

Figure 4—Plots showing blood flow responses to intra-arterial infusion of escalating doses of acetylcholine during the concomitant infusion of saline or L-NMMA in lean (left panel) and obese subjects (right panel). All values are means ± SEM. The *P* values refer to the comparisons of vascular responses to acetylcholine between saline and L-NMMA by two-way ANOVA for repeated measures.

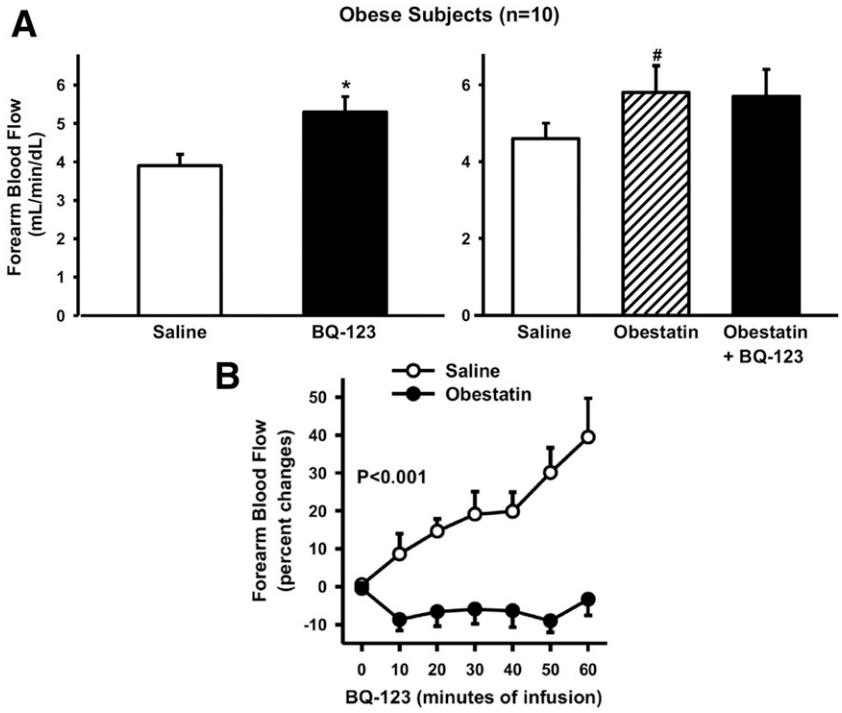

Figure 5—*A*: Bars showing forearm flow values at baseline and after ET_A receptor blockade in obese individuals in the absence (left panel) or the presence (right panel) of exogenous obestatin. All values are means ± SEM. The *P* values refer to the comparisons of vascular responses under different conditions by paired *t* test and one-way ANOVA for repeated measures, followed by the post hoc Holm-Šídák test for multiple comparisons, as appropriate. **P* = 0.002 and #*P* = 0.009 vs. saline. *B*: Plot showing percentage changes in blood flow from baseline in response to intra-arterial infusion of BQ-123 during the concurrent infusion of saline or obestatin (0.8 nmol/min). All values are means ± SEM. The *P* value refers to the comparison of vascular responses under different conditions by two-way ANOVA for repeated measures.

of lean subjects. More importantly, the vasodilator effect of obestatin is substantially preserved in obese individuals, irrespective of their metabolic status, given that their response to obestatin in percentage changes from baseline was only slightly, although significantly, lower than that observed in lean control subjects. We acknowledge, however, that our study recruited considerably fewer lean individuals than obese participants, which might have possibly influenced the magnitude of the difference between the groups in the vascular response to obestatin.

The obestatin-induced vasodilation in both groups was predominantly related to enhanced NO activity, because it was almost completely abolished in the presence of NO synthase inhibition by L-NMMA. The specificity of this effect of obestatin as an NO-related vasodilator in the human circulation is strengthened by the results obtained with infusion of an established endothelium-dependent vasodilator, such as acetylcholine, whose effects substantially mimicked those of obestatin in both lean and obese participants. In this regard, our findings are in close agreement with the observations made by Agnew et al. (9) in isolated vessel preparations of rat aorta and mesenteric artery, where obestatin elicits relaxation attenuated by endothelial denudation and NO synthase inhibition but is unaltered by inhibition of endothelium-derived hyperpolarization. Indirect support to a role of obestatin as a vasodilator in humans also stems from an association study showing that circulating levels of obestatin bear an inverse relationship with blood pressure values in insulin-resistant patients (16). In addition, hypertensive patients, particularly those with obesity, have reduced plasma obestatin levels compared with their normotensive counterparts (17,18), thereby suggesting that obestatin may play a role in blood pressure regulation in humans.

Interestingly, the benefit of obestatin to activate the NO pathway in obese vessels, as seen in the current study, does not seem to diverge from the one previously observed with the sister hormone ghrelin. Thus, we first reported that administration of exogenous ghrelin is able to improve endothelium-dependent vasodilation in the human forearm circulation of patients with obesity-related metabolic syndrome by increasing the bioavailability of NO (19). Those observations have been subsequently expanded by Virdis et al. (20), who observed that ghrelin administration restores NO-mediated vasodilation in patients with essential hypertension by decreasing oxidative stress. Overall, these findings suggest that even though obestatin may oppose some of the biological effects of ghrelin on appetite and metabolism, both products of the ghrelin gene share similar vascular benefits in humans. This apparent contrast may be explained by the fact that although the endocrine actions of ghrelin are mediated by binding of its acylated form to the GHSR1a receptor, some nonendocrine activities, including those on the cardiovascular system, seem predominantly attributable to the action of nonacylated ghrelin on

different receptors (21). Also, the metabolic and cardiovascular effects of obestatin appear to be preferentially mediated by differential domains of the peptide (9,22), thereby bringing an additional level of complexity to the biological actions of these two conjoined peptides. In our investigation, we also measured effluent venous levels of obestatin and ghrelin during the infusion of escalating doses of exogenous obestatin. As expected, circulating obestatin increased progressively during the infusion, whereas ghrelin levels were left unchanged. These findings, therefore, strengthen the specificity of the relation between the observed dose-dependent vasodilator responses and the increased obestatin levels in the bloodstream.

Another important result of our study is that in obese participants, in the presence of exogenous obestatin, blockade of ET_A receptors did not result in any additional vasodilation. This is at odds with the vasodilator response to BQ-123 observed in the same individuals during saline and suggests that ET-1–dependent vasoconstrictor tone was indeed inhibited by obestatin. This finding, observed in both metabolic subgroups, seems of great relevance, because hampering of ET-1–dependent vasoconstriction in the obese vasculature represents an additional benefit of obestatin. Because our study was performed in the intact human circulation, we could not ascertain the precise mechanism underlying the action of obestatin on the ET-1 system. One hypothesis may relate to possible interactions between the obestatin and the ET-1 systems in blood vessels, including an inhibitory action of obestatin at the ET_A receptor level or its downstream signaling pathway. Another, more likely, explanation may involve the observed action of obestatin to enhance NO activity in the obese vasculature. Thus, in addition to its direct role to relax smooth muscle cells, the L-arginine/NO pathway also acts as a modulator of the constrictor forces within blood vessels by inhibiting ET-1 production (23). It is hence conceivable that increased NO activity in our participants after administration of exogenous obestatin might have blocked intravascular production of ET-1. Again, this effect of obestatin is comparable to the one we previously reported in obese patients after administration of ghrelin (13), thereby bolstering the view that these two substances possess equivalent vascular actions.

Several studies have compared circulating levels of obestatin between lean subjects and patients with metabolic disease, with the aim of supporting the potential relevance of this peptide to obesity and diabetes. These studies, however, have yielded inconsistent results. Thus, some have shown decreased concentrations of obestatin in the blood of patients with insulin resistance (16), obesity (24), or type 2 diabetes (25); similarly, studies have reported increased plasma levels of obestatin after weight reduction achieved by bariatric surgery in patients with obesity (26) or type 2 diabetes (27), supporting the notion of an inverse relationship between circulating obestatin and body weight. This view, however, has been undermined by the results of other studies demonstrating that obestatin plasma levels are increased in patients with obesity (28) or metabolic syndrome (29) and unchanged after weight loss induced by gastric surgery (30,31). The precise reasons for these discrepancies are unknown, but in addition to possible differences in the characteristics of the patients studied, variations in the specificity of the methods used for detection of obestatin compared with prepro-ghrelin (32) are another likely contributor. Our study found that circulating obestatin levels are slightly but not significantly lower in obese than in lean participants. The limited number of individuals recruited, however, in addition to the limitations reported above, prevent further inferences on this finding.

Irrespective of all notes of caution, the results of our study clearly indicate that obestatin acts in the human circulation to induce NO-dependent vasodilation, a benefit also present in the obese vasculature where it coexists with inhibition of the ET-1 system. These advantageous vascular effects sum to the previously reported actions of the peptide to improve glucose and lipid metabolism as well as to its inhibitory or neutral role on food intake and gastric empting. Put together, all of these properties make obestatin a much more promising target than ghrelin for cardiovascular disease prevention in obesity and type 2 diabetes, a potential that becomes particularly attractive also in view of the limited availability of current treatments with proven efficacy in this regard (33). The real translational relevance of our current findings, however, still remains to be determined. Among the issues to be addressed, for example, is that obestatin has a short half-life and is rapidly degraded by several proteases located in blood and tissues (34). Development of more stable obestatin analogs resistant to endogenous degradation and providing improved bioactivity, as well as further understanding about its native receptor(s) and the related downstream signaling pathways, may certainly help to fully assess the therapeutic capabilities of this peptide.

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Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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