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Vascular Generation of Tumor Necrosis Factor-α Reduces Nitric Oxide Availability in Small Arteries From Visceral Fat of Obese Patients

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Objectives The aim of this study was to assess whether small arteries from visceral fat of obese patients show a reduced nitric oxide (NO)-dependent relaxation, as compared with lean control subjects, focusing on the role of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α.

Background Visceral obesity is characterized by endothelial dysfunction.

Methods Small arteries from 14 obese (body mass index 48.4 $\pm$ 11 kg/m^2) and 14 control subjects (body mass index 24.9 $\pm$ 2 kg/m^2), dissected after a visceral fat biopsy (laparoscopy), were evaluated on a pressurized micromyograph. Endothelium-dependent relaxation was assessed by acetylcholine. The NO availability, superoxide production, and inflammation were assessed by testing acetylcholine under the nitric oxide synthase (NOS) inhibitor N$^{\omega}$-nitro-L-arginine methylester, tempol (superoxide scavenger), and infliximab (monoclonal anti–TNF-α antibody), respectively. The roles of nicotinamide adenine dinucleotide phosphate oxidase and inducible nitric oxide synthase (iNOS) were assessed by their selective inhibitors apocynin and S-methylisothiourea (SMT), respectively. Vascular superoxide generation (dihydroethidium staining) protein expression of TNF-α and NOS isoforms (Western Blot) and TNF-α localization (immunohistochemistry) were assessed.

Results Vessels from obese patients displayed a blunted relaxation to acetylcholine and a reduced inhibitory effect of N$^{\omega}$-nitro-L-arginine methylester. These alterations were normalized by tempol or infliximab while being partly ameliorated by apocynin and SMT. Vascular superoxide generation was increased ($p < 0.01$) in obese patients. This condition was abrogated by both tempol and infliximab and partly ($p < 0.05$ vs. control subjects) reduced by apocynin or SMT. Enhanced TNF-α and iNOS expression together with increased TNF-α localization in the vascular media were detected.

Conclusions Small arteries from visceral fat of obese patients are characterized by an increased TNF-α production, which reduces NO availability by promoting superoxide generation via nicotinamide adenine dinucleotide phosphate oxidase and iNOS activation. (J Am Coll Cardiol 2011;58:238–47) © 2011 by the American College of Cardiology Foundation

Endothelial dysfunction, secondary to an impaired nitric oxide (NO) availability resulting from increased reactive oxygen species (ROS) generation, is a common alteration of the major cardiovascular risk factors (1,2). Such alteration represents a pathogenic mechanism determining atherosclerotic disease and related cardiovascular events (3). Recently, a key role played by low-grade vascular inflammation on endothelial dysfunction and atherosclerosis emerged (4,5). In particular, the pro-inflammatory cytokine tumor necrosis factor (TNF)-α is involved in reducing vascular NO availability. This action is exerted both indirectly, by induction of ROS generation via nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase or inducible nitric oxide

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synthase (iNOS) activation, and by direct inhibition of endothelial nitric oxide synthase (eNOS) activity (4).

Obesity is a complex chronic disease associated with increased cardiovascular mortality (6). Beyond the concomitance with cardiovascular risk factors—including hypertension, dyslipidemia, and diabetes mellitus—which greatly contribute to accelerate vascular atherosclerosis, obesity is per se responsible for direct detrimental effects on vasculature. In particular, visceral obesity is characterized by reduced endothelial function, an alteration documented in several different vascular districts (7–10). Over the past decade, obesity has been independently associated with a condition of chronic inflammation secondary to an abnormal production of pro-inflammatory mediators, including TNF-α, which might negatively influence vascular reactivity (11,12). More recently, it was shown that local inflammation of perivascular fat might exert a detrimental action on the vascular tone of adjacent small vessels in obese patients (13). These findings support a close interaction between obesity and inflammation and indicate that vasculature is a target of adipose tissue-derived inflammatory cytokines. However, whether low-grade inflammation can be detected at the level of the vasculature from visceral fat is still undetermined. In addition, the molecular mechanisms whereby vascular inflammation might affect vascular endothelial function in humans have not been fully clarified. Accordingly, the present study was designed to assess whether small resistance arteries, isolated from visceral fat in patients with severe obesity, display endothelial dysfunction and the molecular mechanisms involved in such alteration, with particular regard to the putative pathogenic role of vascular TNF-α on ROS generation and NO availability. The possible contribution of vascular NAD(P)H oxidase, iNOS, and xanthine oxidase on ROS generation was also assessed.

Methods

Study population. The study population included 14 patients with severe abdominal obesity (age 39.8 ± 10.2 years) and 14 nonobese control subjects (age 40.8 ± 4.6 years). Obese patients were recruited among 220 consecutive patients referring to the Department of Endocrinology of our university from January 2008 to January 2010 for screening in view of laparoscopic bariatric surgery. The exclusion criteria were: history or clinical evidence of hypertension (blood pressure >140/90 mm Hg); clinical or biochemical evidence of thyroid, adrenal, or gonadal dysfunction; ethanol consumption (more than 60 g or one-half liter of wine/day); hypercholesterolemia; diabetes mellitus; history of congestive heart failure; cardiac and/or cerebrovascular ischemic disease; impaired renal function; smoking history; or menopause. Patients taking cardiovascular or metabolic drugs were also excluded. Control subjects were recruited among patients hospitalized into the Surgery Unit to undergo laparoscopic surgery for cholecystectomy. Exclusion criteria were the same as those adopted for patients, and a body mass index (BMI) >30 kg/m^2. The study protocol was approved by the local ethics committee. All participants were aware of the nature and purpose of the study and gave their written consent.

Anthropometric and biochemical measurements. Sitting blood pressure (average of 3 measurements) was measured with a mercury sphygmomanometer, by means of a standard cuff and a large cuff in lean and obese individuals, respectively. Venous blood samples were taken for measurement of standard hematology, TNF-α (enzyme-linked immunoadsorbent assay commercial kit), and insulin (radioimmunoassay). Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR).

Preparation of small arteries and functional experiments. Small arteries (150 to 300 μm, approximately 2 mm long) were isolated from visceral fat immediately after biopsy sample procurement, performed during laparoscopic surgery, and mounted on 2-glass microcannulae in a pressurized myograph, as previously described (14,15). Endothelium-dependent and endothelium-independent relaxations were assessed by measuring dilatory responses to cumulative concentrations of acetylcholine (ACh) (1 nmol/l to 100 μmol/l, Sigma Chemicals, St. Louis, Missouri) and sodium nitroprusside (0.01 to 100 μmol/l, Sigma), respectively. Vessels were pre-contracted with noradrenaline (1 μmol/l), whose concentration was chosen according to preliminary dose-titration experiments, to establish the threshold concentration able to elicit similar contractions among the experimental groups (data not shown).

Influence of NO availability and ROS production on endothelium-dependent relaxation. To evaluate NO availability and ROS production, curves to ACh were constructed before and after 30-min incubation with the NOS inhibitor N$^{\omega}$-nitro-L-arginine methylester (L-NAME) (100 μmol/l, Sigma) and the antioxidant and superoxide dismutase mimetic tempol (1 mmol/l, Sigma) (16), respectively. To assess whether ROS generation could influence NO availability, ACh was also infused under simultaneous incubation with L-NAME and tempol.

Abbreviations and Acronyms

ACh = acetylcholine

BMI = body mass index

DAB = 3,3 diaminobenzidine tetra-hydrochloride

DHE = dihydroethidium

E_{max} = maximal acetylcholine- and sodium nitroprusside-induced responses

eNOS = endothelial nitric oxide synthase

HOMA-IR = homeostasis model assessment of insulin resistance

iNOS = inducible nitric oxide synthase

L-NAME = N$^{\omega}$-nitro-L-arginine methylester

NAD(P)H = nicotinamide adenine dinucleotide phosphate

NO = nitric oxide

PBS-T = Tween-20 phosphate-buffered saline

ROS = reactive oxygen species

SMT = S-methylisothiourea

TNF = tumor necrosis factor

WC = waist circumference

WHR = waist/hip ratio

Involvement of TNF-α in endothelium-dependent relaxation. To ascertain the contribution of TNF-α to endothelial dysfunction, in small vessels from obese patients and control subjects, a curve to ACh was constructed after 30-min incubation with the anti–TNF-α monoclonal antibody infliximab (100 μmol/l, Schering-Plough [Merck], Whitehouse Station, New Jersey). Previous in vitro studies established that such infliximab concentration is able to suppress the biological activity of TNF-α (17). Acetylcholine was infused in arteries from obese patients under simultaneous incubation with L-NAME and infliximab, to assess the possibility that TNF-α could affect NO availability.

Influence of NAD(P)H oxidase and iNOS in endothelium-dependent relaxation. To evaluate the influence of NAD(P)H oxidase and iNOS on endothelial function, ACh was tested on further sets of small arteries from obese patients, after 30-min incubation with the NAD(P)H oxidase inhibitor apocynin (10 μmol/l, Fluka, Buchs, Switzerland) or the selective iNOS inhibitor S-methylisothiourea (SMT) (100 μmol/l, Sigma). Finally, to assess whether NAD(P)H oxidase or iNOS were involved in decreasing NO availability, ACh was tested during simultaneous incubation with L-NAME plus apocynin and/or SMT. Concentrations of apocynin and SMT were selected according to previous studies reporting the concentrations able to inhibit the respective enzymatic activities (15,18).

Detection of vascular superoxide anion generation. The in situ production of superoxide anion was measured by means of the fluorescent dye dihydroethidium (DHE) (Sigma), as previously described (15). Three slides/segment were analyzed simultaneously after incubation with tempol (100 μmol/l), infliximab (100 μmol/l), apocynin (100 μmol/l), SMT (100 μmol/l), the xanthine oxidase inhibitor allopurinol (100 μmol/l), or Krebs solution at 37°C for 30 min. Krebs-N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid buffer containing 2 μmol/l DHE was then applied into each section and evaluated under fluorescence microscopy. The percentage of arterial wall area stained with the red signal was evaluated with imaging software (McBiophotonics Image J, National Institutes of Health, Bethesda, Maryland).

Western blot analysis of TNF-α, iNOS, and eNOS. Specimens of mesenteric arteries were homogenized in radioimmunoprecipitation assay buffer with a polytron homogenizer and centrifuged at 20,000 rpm for 15 min at 4°C. The resulting supernatants were separated from pellets and stored at −20°C. Aliquots of 50 μg of protein were separated by electrophoresis on 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The blots were then blocked for 2 h with 1% bovine serum albumin in Tween-20 phosphate-buffered saline (PBS-T) and incubated overnight at room temperature with a primary antibody raised against eNOS, iNOS (BD Transduction Laboratories, San Jose, California), TNF-α (Santa Cruz Biotechnology, Santa Cruz, California), or beta-actin (Sigma-Aldrich, Milano, Italy). After repeated washings with PBS-T, a peroxidase-conjugated secondary antibody was added for 1 h at room temperature. After repeated washings with PBS-T, immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon, Millipore, Massachusetts) and exposed to Kodak Image Station 440 (Eastman Kodak Company, Rochester, New York) for signal and densitometric image analysis. To ensure equal loading and accuracy of changes in protein expression, protein levels were normalized to beta-actin.

Immunostaining of TNF-α. Tissue specimens were immediately fixed in cold neutral 4% formaldehyde and then paraffin-embedded. Eight-micrometer-thick sections were microwaved; immunostained by rabbit anti–TNF-α polyclonal antibodies (Abcam, Cambridge, Massachusetts); and treated with biotinylated anti-rabbit immunoglobulins, peroxidase-labeled streptavidin complex (Vector Laboratories, Burlingame, California), and 3,3 diaminobenzidine tetra-hydrochloride (DAB) (Dakopatts, Glostrup, Denmark), as previously reported (19). Negative controls were obtained by substituting the primary antibody with pre-immune rabbit serum. Endogenous peroxidases and avidin-binding activity were assayed by incubating slides with DAB alone or with streptavidin horseradish peroxidase complex/DAB, respectively. The slides were examined under light microscope at 400× magnification equipped with the digital camera DFC480 (Leica, Cambridge, United Kingdom). In the whole artery section, the percentage of immunoreactive tissue was evaluated on representative wall microscopic fields with image analysis software (McBiophotonics Image J) and normalized to the total area examined.

Data analysis. Results are presented as mean ± SEM and analyzed by repeated measures analysis of variance, followed by Student-Newman-Keuls test, or by unpaired Student *t* test where appropriate. To highlight obesity-related factors playing a major role as determinants of the impaired NO-dependent relaxation, univariate and multivariate linear regression analyses were performed between the inhibitory effect of L-NAME on the maximal relaxant response to ACh and BMI, waist circumference (WC), waist/hip ratio (WHR), HOMA-IR, insulin, or TNF-α plasma levels. Finally, in the whole population, a linear correlation between circulating TNF-α levels and the extent of its vascular staining was assessed. A value of $p < 0.05$ was considered statistically significant. Maximal ACh- and sodium nitroprusside-induced responses (E_{max}) were calculated as maximal percentage increments of lumen diameter.

Results

Obese patients showed higher BMI, WC, and WHR values compared with control subjects, whereas blood pressure values, lipid profile, and plasma glucose were similar in both groups. Plasma TNF-α, insulin, HOMA-IR, and leptin

values were also higher in obese patients compared with control subjects (Table 1).

Evaluation of NO availability and influence of ROS production on endothelium-dependent relaxation. Small arteries from obese patients showed significantly ($p < 0.001$) impaired relaxation responses to ACh, compared with control subjects (Figs. 1A and 1B), whereas relaxations to sodium nitroprusside were similar in obese (E_{max}: 94.5 ± 1.1%) and control subjects (E_{max}: 95.1 ± 1.4%). In arteries from control subjects, the relaxation to ACh was significantly blunted by L-NAME (Figs. 1A and 2), and tempol did not affect the response to ACh or the inhibitory effect of L-NAME on ACh-induced relaxation (Fig. 1A). By contrast, in small vessels from obese patients, the inhibitory effect exerted by L-NAME on relaxation to ACh was significantly lower compared with control subjects (Figs. 1B and 2). In these vessels, tempol normalized the endothelium-dependent relaxation and restored the inhibitory effect of L-NAME on ACh (Figs. 1B and 2).

Involvement of TNF-α in endothelium-dependent relaxation. In the control group, relaxation to ACh (E_{max}: 95.5 ± 1.8%) was not modified by infliximab (E_{max}: 95.1 ± 1.4%). By contrast, in vessels from obese patients, infliximab normalized the relaxation to ACh and also restored the inhibitory effect of L-NAME on ACh (Fig. 1C). Of note, in the presence of infliximab, the blunting effect of L-NAME on ACh was similar to that elicited by tempol and no longer different from that recorded in control subjects (Fig. 2).

Influence of NAD(P)H oxidase and iNOS on endothelium-dependent relaxation. In arteries from obese patients, apocynin greatly improved but did not normalize the relaxation to ACh and partly ameliorated the inhibition by L-NAME on ACh-induced responses (E_{max} ACh: 62.1 ± 2.4%; ACh+L-NAME: 53.6 ± 2.2%; ACh+apocynin: 78.7 ± 0.5%; ACh+L-NAME+apocynin: 59.7 ± 1.0%)

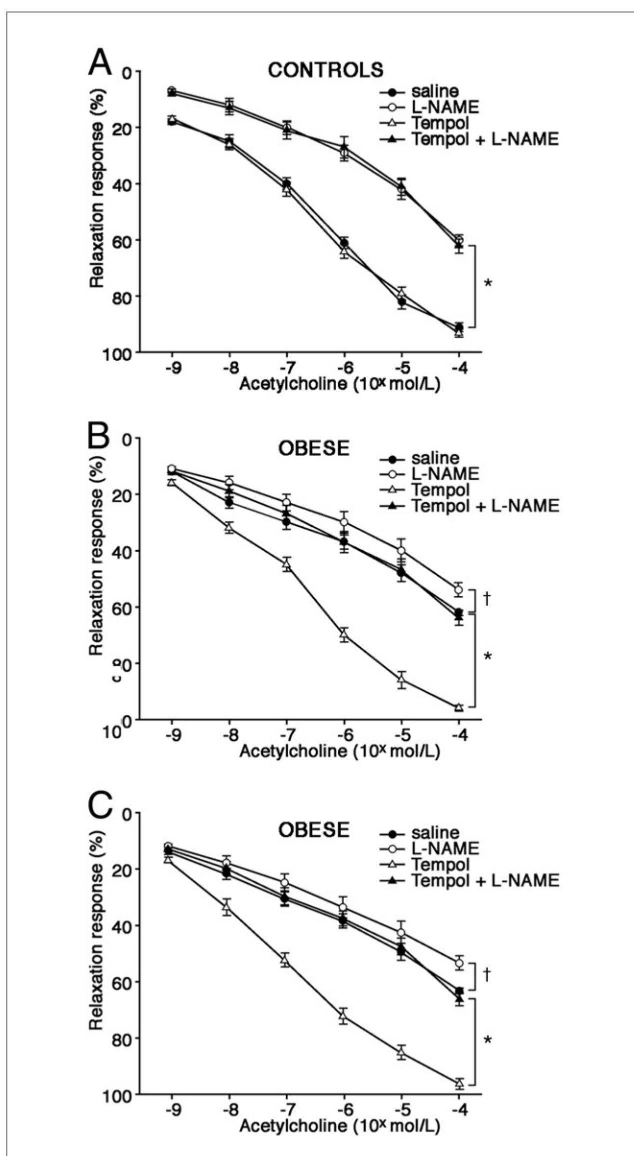

Figure 1 **Endothelium-Dependent Relaxation in Small Arteries From Obese and Control Subjects**

(A) Relaxations to acetylcholine (ACh) in control subjects ± Nω-nitro-L-arginine methylester (L-NAME), tempol, or both. **(B)** Relaxations to ACh in obese patients ± L-NAME, tempol, or both. **(C)** Relaxations to ACh in obese patients ± L-NAME, infliximab, or both. Each **point** is the mean of experiments ± SEM. *$p < 0.001$; †$p < 0.05$.

Table 1 **Clinical Characteristics and Anthropometric Values of Study Populations**

Parameter	Obese (n = 14)	Control Subjects (n = 14)
Body mass index (kg/m^2)	46.3 ± 10*	24.5 ± 2
Waist circumference (cm)	134.6 ± 14.7*	92.0 ± 4.6
Waist/hip	1.06 ± 0.08*	0.93 ± 0.03
Systolic blood pressure (mm Hg)	122.6 ± 9.7	124.5 ± 6.2
Diastolic blood pressure (mm Hg)	80.0 ± 8.7	78.9 ± 3.4
TNF-α (pg/ml)	14.7 ± 6.2*	8.6 ± 6.5
Total cholesterol (mg/dl)	213 ± 17	203 ± 18
HDL cholesterol (mg/dl)	55 ± 13	52 ± 8
Triglycerides (mg/dl)	120 ± 44	126 ± 18
LDL cholesterol (mg/dl)	133 ± 19	126 ± 21
Fasting glucose (mg/dl)	99 ± 14	89 ± 6
Fasting insulin (μU/ml)	13.6 ± 4.2*	7.2 ± 1.5
HOMA-IR	3.6 ± 1.3*	1.6 ± 0.4
Leptin (ng/ml)	22.3 ± 5.2*	9.3 ± 1.9

*$p < 0.05$ versus control subjects.
HDL = high-density lipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; LDL = low-density lipoprotein; TNF = tumor necrosis factor.

(Fig. 2). In these vessels SMT partly potentiated the relaxation to ACh and the inhibitory effect of L-NAME on Ach (E_{max} ACh: 63.2 ± 1.1%; ACh+L-NAME: 52.5 ± 1.3%; ACh+SMT: 80.2 ± 0.8%; ACh+L-NAME+SMT: 59.5 ± 0.9%), similarly to results obtained with apocynin (Fig. 2). When simultaneously infused, apocynin and SMT normalized the relaxation to ACh (E_{max}: 96.7 ± 2.1%) and fully restored the inhibitory effect of L-NAME on ACh (Fig. 2).

Determinants of NO availability in small arteries from the whole study population. Univariate analysis showed a significant inverse correlation between the inhibitory effect

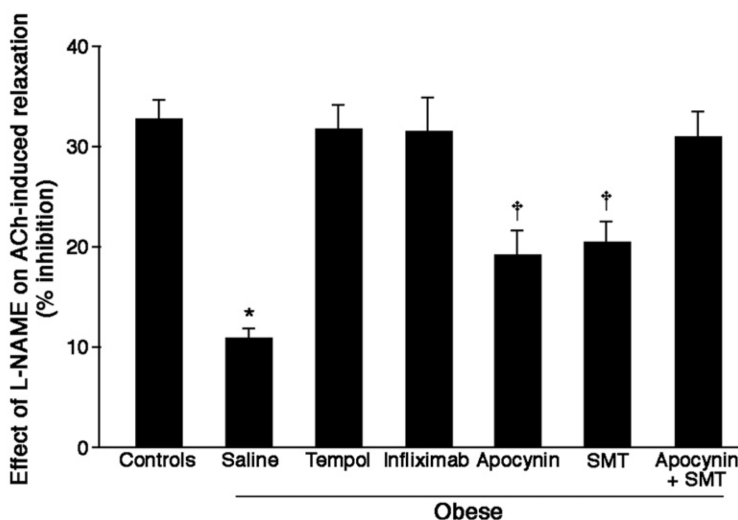

Figure 2 **Effects of L-NAME on Endothelium-Dependent Relaxation in Obese Patients and Control Subjects**

Percentage inhibitory effect of L-NAME on maximal response to ACh ± tempol, infliximab, apocynin, S-methylisothiourea (SMT), or apocynin plus SMT. Each **column** represents the mean of experiments ± SEM. *$p < 0.001$ versus other groups; †$p < 0.05$ versus control subjects, tempol, infliximab, and apocynin plus SMT. Abbreviations as in Figure 1.

of L-NAME on the maximal relaxation to ACh—taken as an indirect index of NO availability—and BMI, WC, WHR, HOMA-IR, insulin, or TNF-α plasma levels (Fig. 3). In multivariate analysis, obesity indexes were the only independent predictors of reduced NO availability (WC: $r^2 = 0.81$; $p < 0.001$; BMI: $r^2 = 0.54$; $p < 0.01$; WHR: $r^2 = 0.39$; $p < 0.05$). Of note, such analyses involved the whole population, because of the linearity of such correlations, without a threshold value.

Analysis of vascular superoxide anion generation. In small vessels from obese patients, DHE analysis revealed a dramatic increase in the superoxide anion production, compared with control arteries (Fig. 4). The superoxide production was abrogated by incubation with infliximab or tempol and partly decreased by apocynin or SMT. Incubation with allopurinol was devoid of any effect (Fig. 4).

Western blot analysis of TNF-α, iNOS, and eNOS. Small vessels from obese patients showed a significantly higher expression of TNF-α as compared with control subjects. Similarly, iNOS protein expression was significantly higher in the obese group than control subjects. The eNOS expression did not differ in the 2 study groups (Fig. 5).

Immunohistochemical analysis of TNF-α. In control arteries, a mild positive immunoreaction was detected within the adventitia and outer smooth muscle cells. By contrast, in vessels from obese patients, there was a marked enhancement of TNF-α immunostaining, mainly localized within the smooth muscle compartment (Fig. 6). Vascular TNF-α staining showed a significant linear correlation with its circulating levels ($r^2 = 0.72$; $p < 0.01$).

Discussion

The first major novel finding of the present study consists in the identification of mechanisms involved in endothelial dysfunction, which affects small resistance arteries in the visceral fat of obese patients. These vessels showed a blunted endothelium-dependent relaxation, together with a reduced inhibitory effect of L-NAME on ACh-induced responses. Such alterations were reversed by the superoxide scavenger tempol. In line with our functional findings, DHE analysis revealed an increased vascular ROS generation, which was abrogated by tempol. The present results agree with—and extend to small resistance arteries from visceral fat—previous demonstrations of endothelial dysfunction in obesity (7–10) and provide the first demonstration that such alterations depend on an increased intravascular superoxide production, which reduces NO availability. To exclude possible confounders, we selected a young obese population, without history of hypertension or evidence of impaired glucose or lipid metabolism. Thus, vascular alterations, as observed in the present study, highlight a specific impact of severe obesity per se on vascular reactivity. This is also supported by our multivariate analysis, indicating obesity indexes as independent variables with a significant negative impact on reduced NO availability. However, our regression analysis was performed in a small group of patients, and a confirmation in a larger population is required.

Role of vascular TNF-α on obesity-associated endothelial dysfunction. Our obese patients showed higher plasma levels of TNF-α than lean control subjects, in line with a previous demonstration (11). Its role as a determinant of

Figure 3 Relationships Between the Effect of L-NAME on Endothelium-Dependent Relaxation and Obesity-Related Factors

Inverse relationship between the percentage inhibitory effect of L-NAME on maximal relaxation to ACh and body mass index (BMI), waist circumference (WC), waist/hip ratio (WHR), homeostasis model assessment of insulin resistance (HOMA-IR), insulin and tumor necrosis factor (TNF)-α plasma values in the whole population of the study. Abbreviations as in Figure 1.

endothelial dysfunction was investigated by the anti–TNF-α monoclonal antibody infliximab. This compound normalized the inhibitory effect of L-NAME on ACh-induced relaxation. Likewise, DHE staining documented that the increased superoxide generation was dramatically reduced by infliximab. These findings provide the first evidence that, in small arteries from the visceral adipose tissue of obese patients, TNF-α plays a major role in maintaining a chronic condition of endothelial dysfunction by stimulating intravascular superoxide generation. Accordingly, a significant up-regulation of TNF-α protein expression in vessels from obese patients was documented. Furthermore, immunohistochemistry indicated a marked up-regulation of TNF-α mainly in the media layer of these vessels. Finally, an inverse relationship between plasma TNF-α levels and the inhibitory effect of L-NAME on ACh emerged. Overall, these observations support the concept that the vascular wall is the source of TNF-α involved in endothelial dysfunction. However, the normalization of vascular dysfunction by infliximab does not necessarily exclude an involvement of additional inflammatory mechanisms, accounting for vascular damage. As well, the possibility that infliximab exerts vascular beneficial effects by interacting with other unknown inflammatory pathways cannot be excluded.

Previous studies in animal models of obesity and in humans have demonstrated that rearrangements in the visceral adipose tissue lead to an increased production of pro-inflammatory cytokines, including TNF-α (12,20). Recent reports have investigated a possible link between TNF-α and obesity-related vascular dysfunction, because the vasculature represents an important target for this cytokine (21). Tesauro et al. (22) documented that infliximab ameliorated peripheral vascular dysfunction during hyperinsulinemia in obese patients by an antioxidant activity. More recently, a potential specific role of inflammation from perivascular adipose tissue in the pathogenesis of vascular dysfunction has been investigated. It was observed

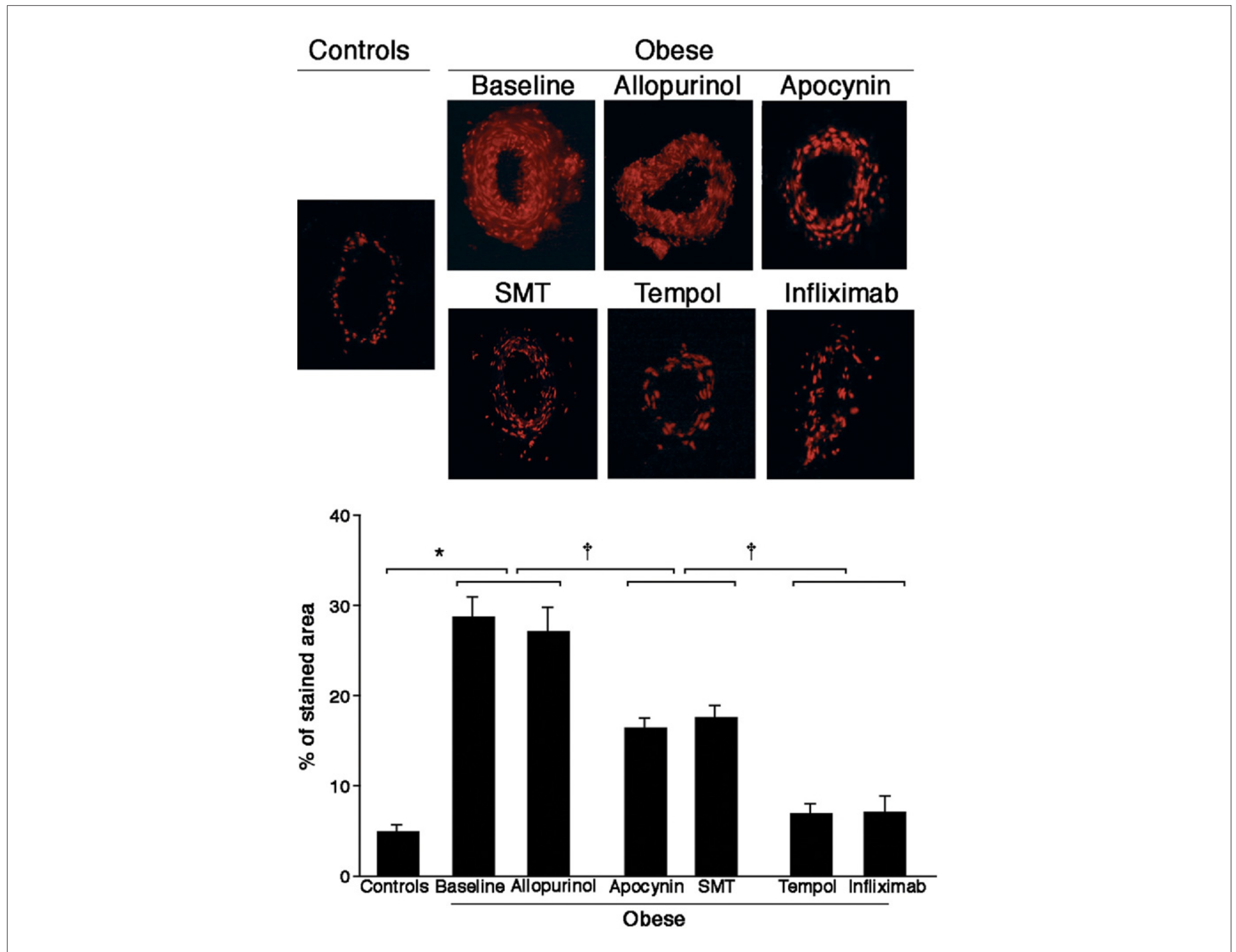

Figure 4 **DHE Staining for Detection of Superoxide Production**

Representative dihydroethidium (DHE) staining **(top)** and quantitative analysis of the red signal **(bottom)** in small arteries from lean control subjects and obese patients at baseline or after incubation with allopurinol, apocynin, S-methylisothiourea (SMT), tempol, or infliximab. Original magnification is 40×. Each **column** represents the mean of 6 experiments ± SEM. *$p < 0.001$; †$p < 0.05$.

that, under healthy conditions, subcutaneous gluteal perivascular fat exerts a protective activity on vasculature, resulting from an anti-contractile property and a favorable influence on NO availability (13) and that such beneficial effects are lost in obese patients as a consequence of several mechanisms, including an increased accumulation of TNF-α in the adipose tissue (13). These observations underscore the role of inflammation as an important promoter of vascular dysfunction and support an important mechanism whereby, in obesity, the adipose tissue damages the surrounding vasculature in a paracrine manner. Our findings add novelty to current knowledge through the demonstration that the vasculature is not only a target but also a source of low-grade inflammation, which in turn—independently of the perivascular adipose tissue—contributes to the pathogenesis of endothelial dysfunction by activating ROS generation. However, our experimental conditions did not allow assessment of whether the small arteries and adipocytes, located within perivascular fat, should be regarded as independent and coexisting sites of TNF-α generation or, by contrast, whether there is a hierarchical relationship or mutual interplay between these 2 districts. Although this issue needs future investigations, the close relationship between vascular TNF-α staining and its increased plasma levels supports the concept of obesity as a generalized inflammatory condition. In this context, the role of insulin deserves a comment. In healthy conditions, insulin induces endothelium-dependent vasorelaxation via NO stimulation (7,23). Thus, it is conceivable that the condition of insulin resistance, which characterized our obese patients, might have interfered with insulin signal transduction, leading to an impaired NO generation. In such a scenario, TNF-α promoted a further exacerbation, resulting from its ability to reduce the density of insulin receptor content at the level of endothelial cells (24).

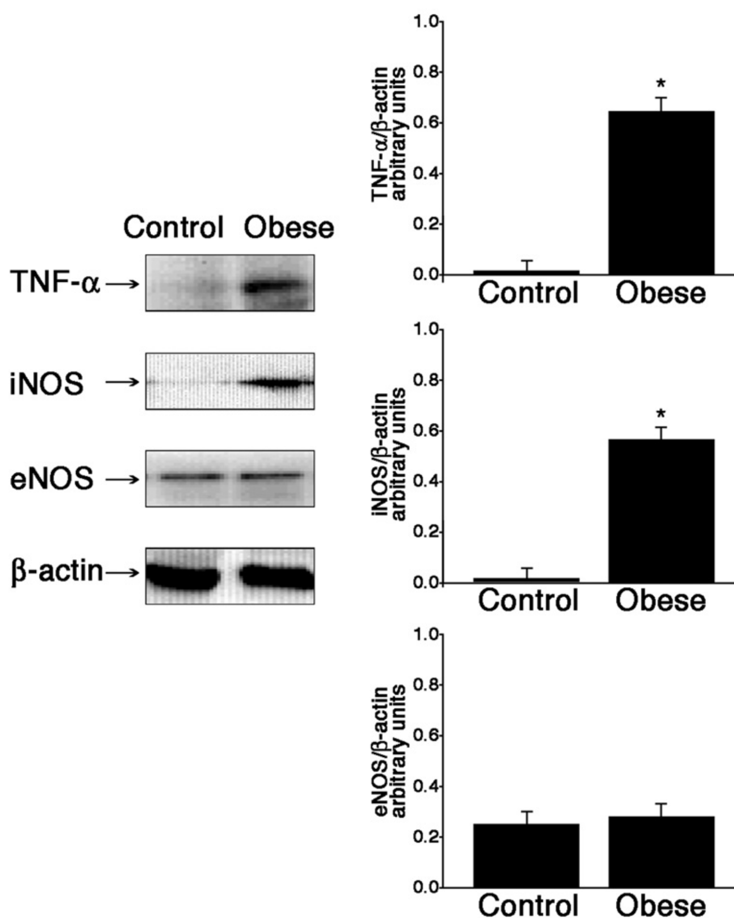

Figure 5 **Western Blot Analysis of TNF-α, iNOS, and eNOS in Small Arteries From Obese Patients and Control Subjects**

Representative blots and column graphs referring to the densitometric analysis of immunoreactive bands. Each **column** represents the mean of 6 experiments ± SEM. $*p < 0.05$. eNOS = endothelial nitric oxide synthase, iNOS = inducible nitric oxide synthase; TNF = tumor necrosis factor.

Pathway(s) involved in TNF-α–induced vascular ROS generation. Toward this aim, we tested apocynin, because of previous studies showing that TNF-α is a relevant stimulus for NAD(P)H oxidase induction (4). The role of iNOS, another enzymatic pathway implicated in ROS production, particularly under inflammatory conditions (25,26), was investigated by its inhibitor SMT. In addition, xanthine oxidase—as an additional endothelial source of superoxide (27)—was assessed by allopurinol. Apocynin partly restored the impaired endothelial function in the obese group. Likewise, SMT increased but did not normalize the NO availability in vessels from obese patients up to values similar to those obtained by apocynin application. The DHE staining showed, in line with these results, that apocynin as well as SMT reduced only in part superoxide generation in arteries from obese individuals. On the basis of these findings, it is conceivable that both NAD(P)H oxidase and iNOS are the 2 major enzymatic pathways that, once simultaneously activated by TNF-α, mediate vascular ROS production in obesity, whereas xanthine oxidase does not seem to be implicated.

Vascular NOS isoforms expression in obesity. Our Western blot analysis revealed an up-regulation of iNOS in arteries from the obese group, in concomitance with a preserved eNOS protein expression. These findings, which are being reported for the first time in human vessels, are in apparent contrast with previous data showing an inhibiting effect of TNF-α on eNOS expression (24,28,29). The unmodified eNOS expression observed in our study suggests that the reduced NO availability secondary to oxidant excess represents a major mechanism accounting for endothelial dysfunction in an obese population. This proposal agrees with a previous demonstration of increased ROS generation in obese patients, an excess reversed by weight loss (30). In addition, we cannot exclude that iNOS—beyond its role as a source of oxidative stress—might at least in part contribute to NO generation, which in turn is rapidly inactivated by ROS.

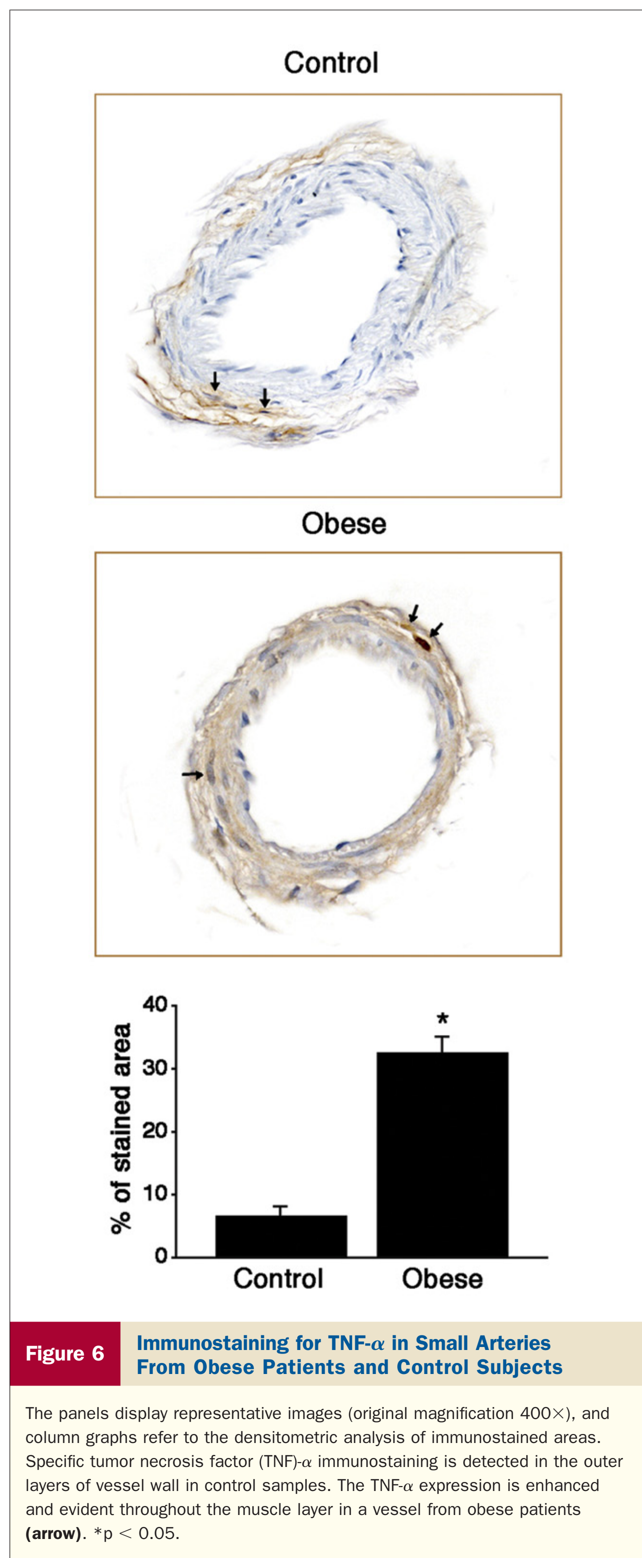

Figure 6 **Immunostaining for TNF-α in Small Arteries From Obese Patients and Control Subjects**

The panels display representative images (original magnification 400×), and column graphs refer to the densitometric analysis of immunostained areas. Specific tumor necrosis factor (TNF)-α immunostaining is detected in the outer layers of vessel wall in control samples. The TNF-α expression is enhanced and evident throughout the muscle layer in a vessel from obese patients **(arrow)**. $*p < 0.05$.

Conclusions

We have demonstrated that small resistance arteries, isolated from the visceral adipose tissue of patients with severe obesity, are characterized by a marked endothelial dysfunction caused by a reduced NO availability. This alteration results from an increased vascular production and biological activity of TNF-α, which promotes superoxide generation via both NAD(P)H oxidase and iNOS activation.

We recognize that any extrapolation from the present to other vascular districts requires caution, because the autocrine/paracrine functions of endothelium can vary, depending on the anatomical location of blood vessels. Nevertheless, these findings, while strengthening the concept that obesity is an inflammatory condition, identify the small vessels of visceral fat as important sources of low-grade inflammation and oxidative stress that—together with the perivascular adipose tissue (31)—might directly contribute to the local development of insulin resistance, which characterizes obese patients.

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