Aging Modulates the Influence of Arginase on Endothelial Dysfunction in Obesity

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Objective**—**Arginase can reduce NO availability. In this study, we explored arginase as a determinant of endothelial dysfunction in small arteries from obese patients and its relationship with aging and microvascular remodeling.

Approach and Results**—**Small arteries were dissected after subcutaneous fat biopsies and evaluated on a pressurized micromyograph. Endothelium-dependent vasodilation was assessed by acetylcholine, repeated under L-NAME (N^G-nitro-L-arginine-methyl ester), N(ω)-hydroxy-nor-L-arginine (arginase inhibitor) and gp91ds-tat (NADPH [nicotinamide adenine dinucleotide phosphate oxidase] oxidase inhibitor) in vessels from young (age <30 years) control and obese and old (>30 years) control and obese subjects. Media-lumen ratio and amount of vascular wall fibrosis were used as markers of vascular remodeling. Amount of vascular superoxide anions and NO production were determined with immunofluorescence, whereas arginase expression was quantified using Western blot and quantitative polymerase chain reaction. Obese and older age groups had lower vascular NO, as well as higher media-lumen ratio, wall fibrosis, intravascular superoxide, and blunted inhibitory effect of L-NAME on acetylcholine versus controls and younger age groups. N(ω)-hydroxy-nor-L-arginine restored the acetylcholine-induced vasodilation in young and, to a lesser extent, in old obese subjects. This effect was abolished by addition of L-NAME. Gp91ds-tat increased the vasodilatory response to N(ω)-hydroxy-nor-L-arginine in old obese. Superoxide anions and arginase I/II levels were higher in the vascular wall of obese versus controls.

Conclusions**—**Arginase contributes to microvascular endothelial dysfunction in obesity. Its impact is reduced by aging because of higher levels of vascular oxidative stress. Obesity is accompanied by accelerated microvascular remodeling, the extent of which is related to the amount of arginase in the vascular wall.

Visual Overview**—**An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38: 2474-2483. DOI: 10.1161/ATVBAHA.118.311074.)**

Key Words: aging ■ arginase ■ endothelial function ■ microvascular ■ obesity ■ superoxide

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Obesity represents a major public health concern, as it is associated with high morbidity and mortality because of several chronic diseases.[1] On the cardiovascular system, obesity accelerates atherosclerosis progression, inducing pathophysiological changes that are detectable already from young adults and are similar to those observed in aging.[2] Among these, a reduced NO availability represents one of the earliest vascular alterations observed in obesity[3,4] and leads to altered vascular homeostasis and accelerated remodeling.

The enzyme eNOS (endothelial NO synthase) is a key regulator of NO availability, as its activity represents the main source of vascular NO. A reduced concentration of the eNOS substrate L-arginine can impair NO production,[5] ultimately leading to endothelial dysfunction.[6] Arginase is a manganese metalloenzyme that hydrolyzes l-arginine to urea and l-ornithine. It exists in 2 distinct isoforms, arginase I and II, both of which are widely expressed in many tissues, including the cardiovascular system. An increased concentration or activity of arginase may cause eNOS uncoupling, resulting in reduced NO production and endothelial dysfunction.[7,8] Hyperactivity of arginase might also promote vascular remodeling ,[9–11] inducing vascular endothelial inflammation and senescence,[12] as well as leading to production of undesired metabolites within the vascular wall that might support smooth muscle cell proliferation and collagen synthesis.[9,11] As reduced NO availability and accelerated vascular aging are key mechanisms of vascular diseases associated with obesity, animal experiments have explored the potential contribution of arginase I and II

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Nonstandard Abbreviations and Acronyms

eNOS	endothelial NO synthase
ICAM	intercellular adhesion molecule-1
norNOHA	N(ω)-hydroxy-nor-L-arginine
TNF-α	tumor necrosis factor α
VCAM	vascular adhesion molecule-1

to the obesity-related endothelial dysfunction and vascular remodeling .[13–15] Only a small study, however, described a possible influence of arginase on endothelial function of insulin-resistant, morbidly obese patients.[15]

Reduced NO availability is also detected in conditions of increased intracellular concentration of reactive oxygen species (ROS). One of the most important sources of intracellular ROS is the enzyme NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, which is upregulated in obesity and aging .[3,16–18] Recent evidence suggests that aging may exacerbate the obesity-induced upregulation of this enzyme within the vascular wall.[19] Thus, the reduced NO availability detected in subjects with obesity might result from both a reduced activity of the eNOS and an increased concentration of intracellular ROS.

In this study, we explored the combined influence of arginase and ROS on endothelial function of subjects affected by obesity. As the influence of ROS on obesity-related endothelial dysfunction might increase with aging, we also assessed whether the contribution of arginase to the obesity-related endothelial dysfunction is reduced in older obese subjects because of higher activity of the NADPH oxidase.

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Materials and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Population

In a case-control design, a total of 36 obese patients and 31 controls were included in the study. Obese participants were consecutively recruited among patients referring to the Department of Clinical and Experimental Medicine of the University of Pisa for a complete clinical evaluation preceding laparoscopic bariatric surgery. Control subjects were recruited among consecutive patients referring to the Department of Surgery of the University of Pisa to undergo elective laparoscopic cholecystectomy because of gallbladder stones. The exclusion criteria for obese subjects were age >60 years, history or clinical evidence of hypertension (blood pressure >140/90 mm Hg), clinical or biochemical evidence of diabetes mellitus or endocrine (thyroid, adrenal, or gonadal) dysfunction, sustained ethanol consumption (>60 g/d), dyslipidemia, smoking habits, renal or liver impairment, menopausal status, and any documented cardiovascular disease. Patients assuming any chronic pharmacological therapy were also excluded. Exclusion criteria for the control subjects were the same as those adopted for obese patients, with the addition of a body mass index >27 kg/m^2. For all participants, a complete medical history was obtained via interview (including smoking history and current or previous use of medications). Blood pressure and heart rate were measured with an oscillometric device, 3×, with the participant in a seated position, after 5 minutes resting in a quiet room. The average of the 3 readings was used for the analyses. High and weight were measured with the participant wearing light clothes and without shoes. The body mass index was calculated as weight (kg) divided by squared height (m^2). All human studies and handling of human material was in accordance to the declaration of Helsinki. The protocol was approved by the local Ethical Committee (protocol no. 12 589), and each participant gave written informed consent to the study.

Table 1. Characteristics of the Study Population Stratified by BMI Group

	Control (n=31)	Obese (n=36)	*P* Values
Age, y	36±11	37±10	NS
Sex (male)	52%	47%	NS
BMI, kg/m^2	25.95±2.38	46.70±5.81	<0.001
Systolic blood pressure, mm Hg	125±7	123±8	NS
Diastolic blood pressure, mm Hg	78±4	79±6	NS
Heart rate, bpm	69±5	79±7	0.039
Creatinine, mmol/L	73.17±13.80	71.83±16.50	NS
Total cholesterol, mmol/L	4.90±0.5	4.8±0.6	NS
HDL cholesterol, mmol/L	1.30±0.2	1.20±0.3	0.006
Triglycerides, mmol/L	1.35±0.35	1.39±0.31	NS
LDL cholesterol, mmol/L	2.94±0.55	2.92±0.51	NS
Fasting plasma glucose, mmol/ L	4.50±0.5	5.0±0.4	NS
Malondialdehyde, µmol/L	1.29±0.20	1.35±0.25	NS
TNF-α, pg/mL	8.5±0.84	14.1±1.36	<0.001
Media-lumen ratio	6.00±0.54	10.48±3.01	<0.001
Media cross-sectional area, µm^2	9892.81±1676.37	17160.67±5340.29	<0.001

Results are expressed as mean±SD or percentage values for categorical variables. BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, nonsignificant; and TNF-α, tumor necrosis factor α.

Biochemical Measurements

Fasting blood samples were collected from each patient before the surgical procedure. Plasma glucose, total serum cholesterol, triglycerides, and HDL (high-density lipoprotein)-cholesterol levels were assessed using standard procedures, whereas LDL (low-density lipoprotein)-cholesterol levels were calculated according to the Friedewald formula.[20] Circulating levels of TNF-α (tumor necrosis factor α) were measured in plasma using an ELISA kit (R&D systems Minneapolis, MN), whereas malondialdehyde was measured in plasma using a spectrophotometric assay (Bioxytech LPO-586; OXIS International, Inc, CA).

Preparation and Functional Experiments of Small Arteries

Small arteries were isolated from biopsies of subcutaneous fat taken during surgical procedures and mounted in a pressurized myograph as previously described.[9,21] Media thickness and lumen diameter were measured in 3 different points from each small artery to obtain the media-lumen ratio (M/L). Media cross-sectional area (MCSA) was obtained by subtraction of the internal from the external cross-sectional areas using external plus lumen diameters as previously described.[22] Endothelium-dependent and endothelium-independent relaxations were assessed by cumulative concentrations of acetylcholine (ACh, 0.001–100 µM; Sigma-Aldrich SRL, Milano, Italy) and

Table 2. Characteristics of the Study Population Stratified by Age and Obesity Groups

	Control <30 y old (n=15)	Control >30 y old (n=16)	Obese <30 y old (n=15)	Obese >30 y old (n=21)
Age, y	26±2	45±8*	27±3	44±7†
Sex (male)	46%	56%	40%	52%
Body mass index, kg/m²	24.60±1.92	27.21±2.10	45.01±7.04‡	47.91±4.54§
Systolic blood pressure, mm Hg	126±7	123±7	121±8	124±7
Diastolic blood pressure, mm Hg	78±4	77±4	78±5	79±6
Heart rate, bpm	71±5	67±5	78±5‡	79±7§
Creatinine, mmol/L	67.92±11.93	77.85±13.96	69.66±17.09	73.37±16.30
Total cholesterol, mmol/L	4.9±0.5	5.0±0.5	4.5±0.6	4.9±0.5
HDL cholesterol, mmol/L	1.3±0.2	1.3±0.2	1.2±0.2	1.3±0.3
Triglycerides, mmol/L	1.33±0.49	1.37±0.14	1.52±0.38	1.30±0.21
LDL cholesterol, mmol/L	2.87±0.61	3.01±0.50	2.63±0.57	3.12±0.36†
Fasting plasma glucose, mmol/L	4.4±0.3	4.6±0.6	4.9±0.3‡	5.1±0.4§
Malondialdehyde, μmol/L	1.20±0.20	1.35±0.17	1.25±0.23	1.42±0.25
TNF-α, pg/mL	6.70±2.72	9.90±4.80	10.93±4.04	16.19±5.97§
Media-lumen ratio	5.49±0.23	6.47±0.20*	7.26±0.50‡	12.79±1.52†§
Media cross-sectional area, μm²	9080.47±1413.82	10654.38±1573.61*	11994.27±2238.05‡	20850.95±3476.14†§

Results are expressed as mean±SD or percentage values for binary variables. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and TNF-α, tumor necrosis factor α.

*Significant differences between young and old control groups.

†Significant differences between young and old obese groups.

‡Significant differences between young obese and young control groups.

§Significant differences between old obese and old control groups.

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sodium nitroprusside (0.01–100 μM; Sigma-Aldrich SRL, Milano, Italy) in vessels precontracted with norepinephrine (1 μM; Sigma-Aldrich SRL, Milano, Italy).

NO availability and the contribution of arginase and NADPH oxidase to the endothelial dysfunction were investigated by repeating ACh after 30-minute incubation with the NO synthase inhibitor L-NAME (N^G-nitro-L-arginine-methyl ester; 100 μM; Sigma-Aldrich SRL, Milano, Italy), the arginase inhibitor N(ω)-hydroxy-nor-L-arginine (norNOHA, 10 μM; Cayman Chemical, MI), and with the NADPH oxidase inhibitor gp91ds-tat (1 μM; DBA, Milano, Italy). We previously documented that gp91ds-tat can significantly reduce the vascular wall superoxide anion production.[10,23] To confirm that a possible recovery of endothelial function after addition or norNOHA was because of improved function of the eNOS, norNOHA and L-NAME were incubated simultaneously. Finally, to explore the concomitant influence of oxidative stress and arginase to the obesity-related endothelial dysfunction, ACh was repeated after incubation with both gp91ds-tat and norNOHA.

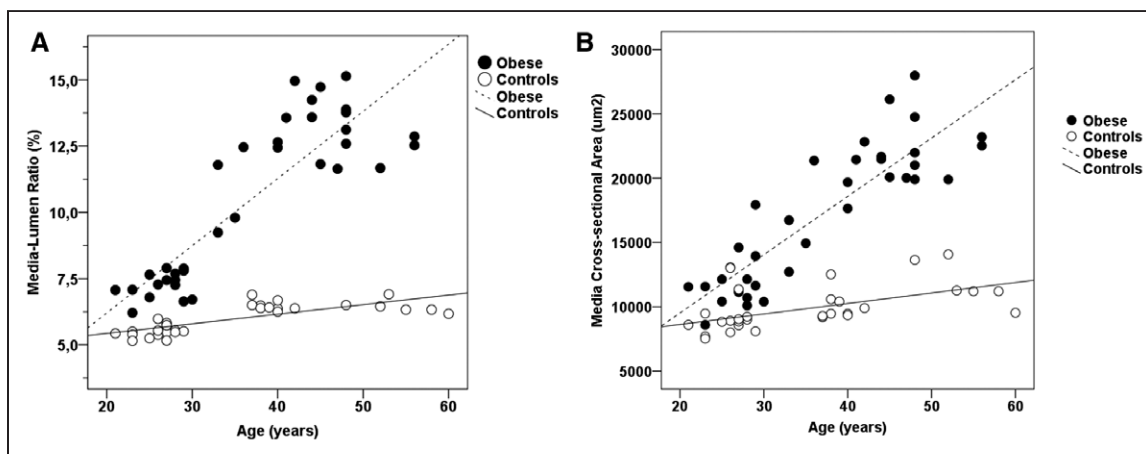

Figure 1. Scatterplots reporting the relationships between parameters of vascular remodeling with age, stratified by obesity groups. **A**, Scatterplot of relationship between age and media-lumen ratio (M/L) in obese (n=36, black circles, dotted line) and control subjects (n=31, white circles, solid line). A significant (age×obesity category) interaction was found (P<0.001), with the obese group showing a faster increase of M/L (r=0.812; P<0.001) per year of age compared with controls (r=0.735; P<0.001). **B**, Scatterplot of relationship between age and media cross-sectional area (MCSA) in obese (n=36, black circles, dotted line) and control subjects (n=31, white circles, solid line). A significant (age×obesity category) interaction was found (P<0.001), with the obese group showing a faster increase of media cross-sectional area (r=0.850; P<0.001) per year of age compared with controls (r=0.649; P<0.001).

Immunofluorescent Assays

Vascular Oxidative Stress

The in situ production of superoxide anion was measured using the fluorescent dye dihydroethidium (DHE; Sigma-Aldrich SRL, Milano-Italy). Three slides per segment were analysed simultaneously after incubation with Krebs solution at 37°C for 30 minutes. Krebs-HEPES buffer containing 2 μM DHE was then applied onto each section and evaluated under confocal microscopy. The percentage of arterial wall area stained with the red signal was evaluated using an imaging software (McBiophotonics Image J; National Institutes of Health, Bethesda, MD). When an increased amount of vascular wall oxidative stress was detected, the DHE staining was repeated after preincubation with the gp91ds-tat, alone and in combination with norNOHA to differentiate the proportion of superoxide anions derived from hyperactivity of NADPH oxidase and eNOS uncoupling because of arginase.

Vascular Wall NO Formation

The in situ production of NO was measured using the fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein (Sigma-Aldrich SRL, Milano, Italy) following a similar protocol than that used for DHE. Three slides per segment were analysed simultaneously. After incubation with Krebs solution at 37°C for 30 minutes, Krebs-HEPES buffer containing 5 μmol/L 4-amino-5-methylamino-2',7'-difluorofluorescein was applied onto each section and evaluated under confocal microscopy. Quantification of the signal was performed following the same approaches and methods used for the DHE staining.

Histochemical Staining

Small arteries from young and old control and obese subjects (n=5 each group) were formalin-fixed, paraffin-embedded, cross-sectioned, and then processed for Sirius Red/Fast Green staining and quantification as previously reported.[22] In brief, representative photomicrographs were taken by a DFC480 digital camera (Leica Microsystems, Cambridge, United Kingdom) applied on a Leica DMRB light microscope. Sirius Red–stained collagen deposition was quantified by a Leica Application Suite software (version 4.0). Our group has previously validated this method to quantify the amount of tissue fibrosis,[24] showing that it provides more reliable results than other histochemical staining methods.[25]

Western Blot Analysis

Western blot analysis was performed to quantify arginase I and arginase II protein expression, as well as levels of malondialdehyde in small arteries of control and obese patients, as reported in the online-only Data Supplement file.

Quantitative Polymerase Chain Reaction Assay for Arginase I and II Expression

Total RNA was isolated from control, as well as from obese subjects using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA). Purified total RNA was transcribed into cDNA with One Step SYBR Prime Script RT-PCR (Takara-Clontech, Kusatsu, Japan). The mRNA expression levels of arginase I and II were determined by real-time polymerase chain reaction using a relative quantification method with GAPDH as an endogenous control.[26] GAPDH, arginase I, and arginase II primers were purchased from Sigma-Aldrich Gene Expression Assay service and were as follows:

GAPDH 5'-ACATCAAGAAGGTGGTGA (F), 5'-GTCAAAGGTGGAGGAGTG (R); ARG 1 5'-GAGAGCTCAAGTGCAGCAAA (F), 5'-TCCCACAGACCTTGGATTCT (R); ARG 2 5'-ATTGATGCATTTGACCCTAC (F), 5'-CCTGTATTGTGTATTTCCTCAG (R). Analyses were performed in 10 μL reaction volume in 48-well plates (Eco Real-Time PCR 48-well plate). Samples were run on Eco Real-Time PCR System (Illumina, Inc, San Diego, CA) using a 2-step program consisting of 10 s at 95°C and 45 s at 58°C for 50 cycles.

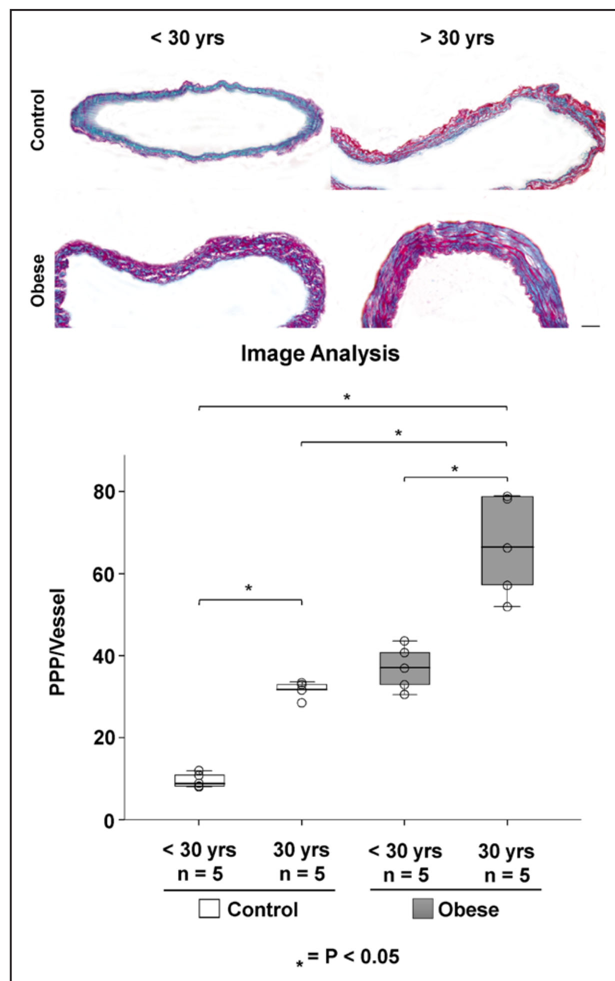

Figure 2. Amount of vascular wall collagen fibers. Representative photomicrographs of sections from cross-sectioned small arteries showing the distribution pattern of Sirius Red–stained collagen fibers and Fast Green–stained noncollagen proteins in the 4 groups of young control, old control, young obese, and old obese subjects (n=5 each). The quantification of collagen fibers is expressed as percentage of positive pixels (PPP) calculated on the vessel area examined. Column graphs display the PPP mean values±SEM. Between-group differences were analysed by 1-way ANOVA using the Tukey test for multiple comparisons. Scale bar=20 μm.

The threshold was set to the geometric phase of the amplification curve. Amplification products were normalized to GAPDH, and the quantification of gene expression was calculated using the formula 2-ΔΔCt.[26]

Statistics

Results were calculated as maximal percentage increments or decrements of lumen diameter from baseline or, when appropriate, from acetylcholine-induced vasodilation from noradrenaline-mediated precontracted vessels. Normality of continuous variables was assessed with the Shapiro-Wilk test and graphically inspected with histograms and quantile-quantile plots. Variables that deviated from normality were log transformed to decrease skewness when used in regression models or parametric tests. Data were presented as mean±SD for continuous variables and as percentages for binary variables. Linear regression was used to assess the association between variables. Initially, analyses were conducted in the whole population and stratified by groups of obese and controls. Then, to explore the combined effects of obesity and age, both the control and obese groups were further stratified according with their age in 2 additional groups: young controls (age <30 years, n=15), aged controls

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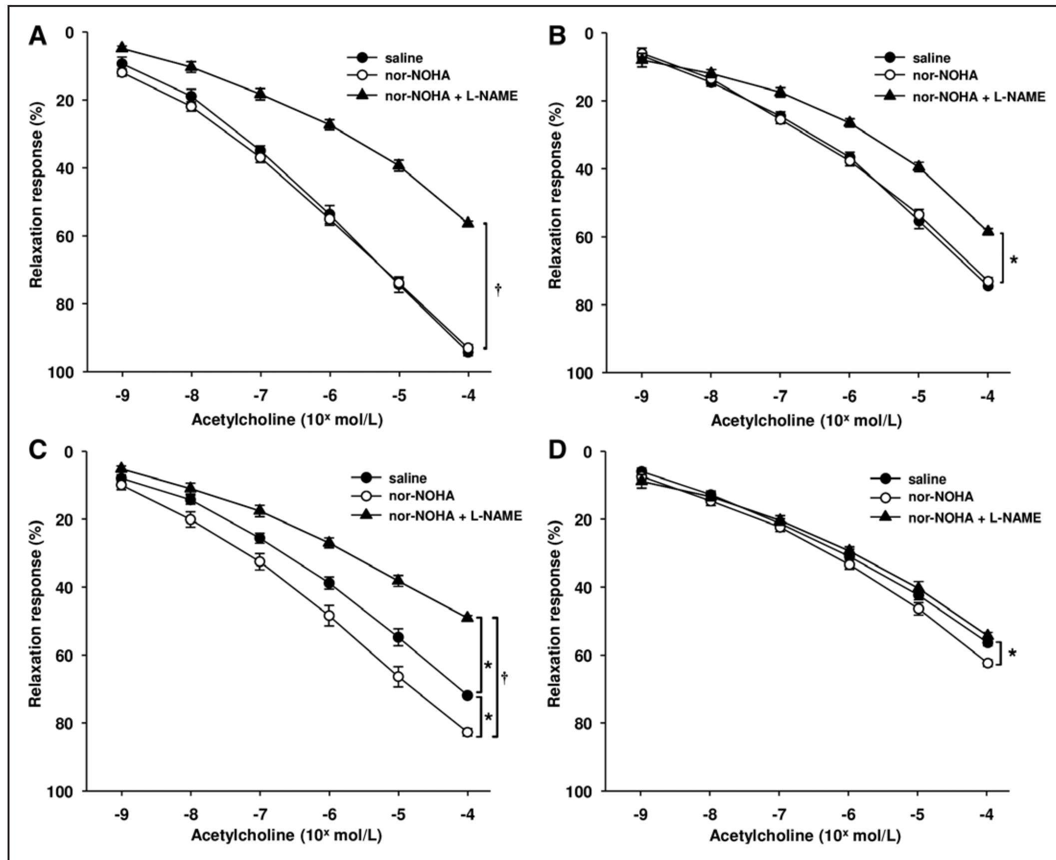

Figure 3. Acetylcholine-induced relaxation with and without inhibition of arginase by N(ω)-hydroxy-nor-L-arginine (norNOHA) and the effect of L-NAME (N^G-nitro-L-arginine-methyl ester). The vasodilatory response to acetylcholine progressively declined from young control to young obese, old control, and old obese groups. Although addition of norNOHA did not improve the response to acetylcholine in young (**A**) and old (**B**) controls, there was a significant improvement in obese groups, which was more evident in young obese (**C**) than in old obese (**D**). The positive effect of norNOHA on the acetylcholine-induced vasodilation in the obese groups was lost when the vessels were preincubated with L-NAME. ANOVA for repeated measures, followed by the Student-Newman-Keuls test, was used to assess differences in the vasodilatory responses induced by preincubation with different substances. †$P<0.001$; * $P<0.05$.

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(age >30 years, n=16), young obese (age <30 years, n=15), aged obese (age >30 years, n=21). One-way ANOVA using the Tukey post hoc test for multiple comparisons and independent samples Student *t* test for pairwise comparisons were used to assess significant differences between groups. ANOVA for repeated measures, followed by the Student-Newman-Keuls test, was used to assess differences in the vasodilatory responses induced preincubation with different vasoactive drugs. A value of $P<0.05$ was considered statistically significant, apart from interaction terms for which a more conservative *P* value <0.1 was considered significant. All analyses were performed using SPSS software (version 20.0; IBM).

Results

Table 1 reports the clinical characteristics of the study population, stratified by groups of body mass index. Obese had a higher heart rate, circulating levels of TNF-α, and lower HDL cholesterol to controls. The same characteristics with the population stratified by age and body mass index categories are reported in Table 2. There was no significant difference of age between young obese and young control, as well as between old obese and old control groups.

Vascular Changes Associated With Aging and Obesity

Aging and obesity had similar effects on the vascular phenotypes. Aging was related to a progressive increase of the M/L (r=0.521; $P<0.001$) and MCSA (r= 0.584; $P<0.001$), and we found evidence of a significant effect modification of these associations by obesity category, so that in obese there was a faster increase of M/L and MCSA per year of increasing age compared with controls (Figure 1A and 1B). In analyses stratified by age and obesity categories, the old obese group showed the highest M/L and MCSA ($P<0.001$ for both compared with all other groups), whereas the young obese had an M/L and MCSA similar to old controls and significantly higher than young controls ($P<0.001$ for M/L and P=0.009 for MCSA; Figure IA and IB in the online-only Data Supplement). In keeping with these structural alterations, the amount of Sirius Red–stained collagen fibers was significantly increased in vessels from old compared with young controls (Figure 2). Young obese showed values of collagen deposition in vascular walls that were comparable with those detected in old controls. The greatest percentage of collagen was found in vessels from old obese patients, which displayed walls filled with collagen fibers (Figure 2). Aging was also associated with worsening endothelial function because of a reduced NO availability, as assessed by the vasodilatory response to ACh (r=−0.509, $P<0.001$) and the inhibitory effect of L-NAME on ACh. In both young and old age groups, obese subjects had a significantly lower vasodilatory response to ACh compared with controls (Figure II in the online-only Data Supplement). Preincubation with L-NAME

significantly blunted the maximal vasodilatory response to ACh in the young control group (ACh, 94.2±1.0%; ACh+L-NAME, 53.5±0.5%; P<0.001) and, to a lesser extent, in the young obese (ACh, 71.8±0.5%; ACh+L-NAME, 52.0±0.5%; P<0.05) and old control (ACh, 74.7±0.4%; ACh+L-NAME, 61.0±1.0%; P<0.05) groups, whereas there was no effect on vessels from the old obese group (ACh, 56.0±1.1%; ACh+L-NAME, 53.3±1.3%; P=nonsignificant). This suggested that NO provided a greater contribution to the endothelial-dependent vasodilation in the young control group, followed by the young obese, old control, and old obese groups. Indeed, the 4-amino-5-methylamino-2',7'-difluorofluorescein assay showed a higher NO generation in the young control group, compared with the young obese, old control and old obese groups, respectively (Figure 5B). Of note, the amount of ACh-induced vasodilation was inversely related with the M/L (r=−0.904; P<0.001) and MCSA (r=−0.840; P<0.001).

The Role of Arginase in Obesity-Induced Endothelial Dysfunction

Inhibition of arginase by preincubation with norNOHA induced a significant improvement of the vasodilatory response to ACh in obese subjects (P<0.001) but not in controls (Figure 3). This improvement was more evident in the young than the old obese group (Figure 3). The addition of L-NAME abrogated the significant improvement of vasodilation obtained with norNOHA observed in the young obese group (P<0.001 for the difference between norNOHA versus L-NAME), suggesting that the addition of norNOHA restored eNOS activity (Figure 3). Vascular levels of arginase I progressively increased by age in both the obese and control groups, and obese had higher levels of arginase compared with control groups (Figure 4). Similar differences were observed for vascular levels of arginase II between obese and controls, and a similar increase with aging was observed in both obese and control groups. The differences in the vascular wall expression of arginase I and II were further confirmed by the quantitative polymerase chain reaction assay, showing significantly higher expression of both enzymes in old compared with young patients and in obese compared with controls (Figure III in the online-only Data Supplement).

The Balance Between Arginase Activity and Vascular Superoxide Anion Generation

Older subjects in both the obese and control groups showed a higher amount of vascular superoxide anions compared with their younger peers (Figure 5A). Results of the immunofluorescent staining with DHE were confirmed by the Western blot analysis, showing a higher concentration of malondialdehyde in the arteries of the old obese compared with the young obese group (Figure 5C). Preincubation with gp91ds-tat (a selective inhibitor of NADPH oxidase) significantly reduced the amount of vascular superoxide anions in old obese subjects. Addition of norNOHA to the gp91ds-tat did not cause further reduction of the DHE signals (Figure 5A), suggesting that only a minimal part of the superoxide anion production observed in this group was because of the uncoupling of the eNOS resulting from higher arginase activity/expression. Based on these results, we tested whether inhibition of NADPH oxidase could restore the response to norNOHA in the old obese group. Preincubation with the gp91ds-tat was accompanied by an improved vasodilatory response to ACh in old obese (P<0.001) and in old controls (P<0.001), whereas this response was reduced in young obese (Figure 6). The addition of norNOHA to gp91ds-tat further increased the ACh-dependent vasodilation in old obese (P<0.001 versus norNOHA or gp91ds-tat alone) and, to a much lower extent, in old controls (Figure 6A and 6B). Absolute relaxation after restoration of the ACh response with norNOHA and gp91ds-tat incubation remained slightly lower in the obese compared with the control groups (P<0.001; Figure IVA in the online-only Data Supplement) and showed a graded inverse relationship with the M/L (r=−0.821; P<0.001; Figure IVB in the online-only Data Supplement) and MCSA (r=−0.771; P<0.001; Figure IVC in the online-only Data Supplement).

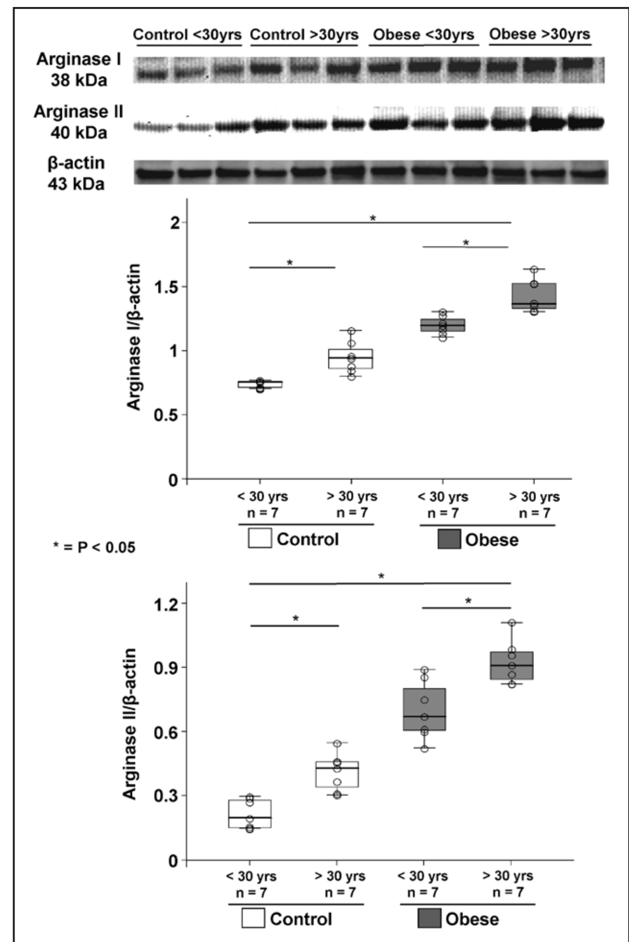

Figure 4. Amount of arginase expression in the vascular wall. Western Blot results showing a progressive increase with aging and obesity of arginase I and II in the vascular wall. Experiments included 7 subjects in each group. Between-group differences in the amount of arginase I and II within the vascular wall were analysed by 1-way ANOVA using the Tukey post hoc test for multiple comparisons.

Discussion

In this study, we demonstrate that arginase is involved in the regulation of NO availability in small vessels from obese and that its influence on endothelial function is modulated

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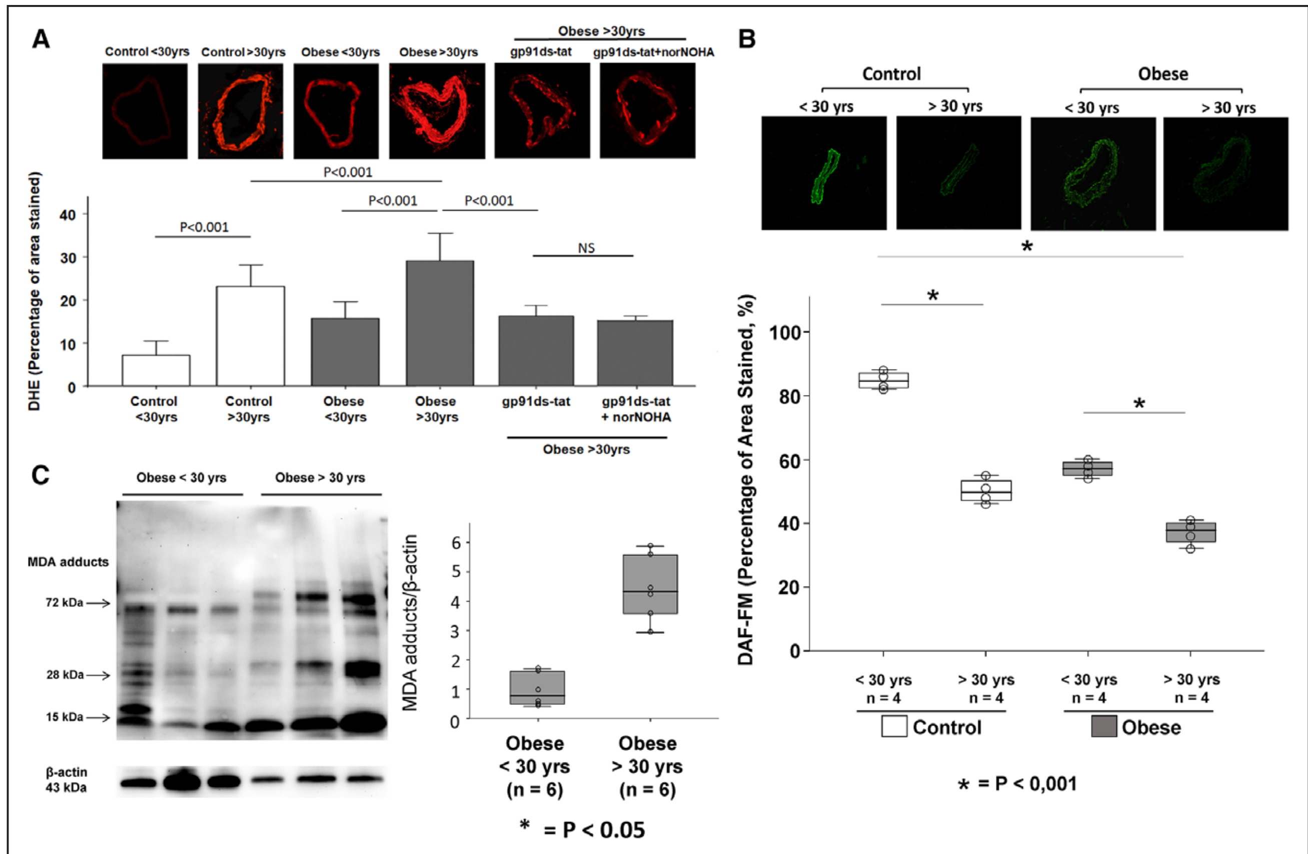

Figure 5. Amount of superoxide anions, malondialdehyde, and NO in the vascular wall of small arteries. **A**, Aged groups had higher superoxide anion production compared with young groups ($P<0.001$) and, in pairwise comparisons, obese groups showed higher amount of vascular superoxide compared with their control peers ($P<0.001$ for both; n=12 in each group except for the young obese group, including 10 subjects). Arteries from 5 subjects were preincubated with gp91ds-tat that induced a significant reduction of the vascular wall production of superoxide anions, without further significant improvement after addition of N(ω)-hydroxy-nor-L-arginine. **B**, NO production in the vascular wall was inversely related to the amount of superoxide anions. Indeed, higher concentrations of NO were detected in the young compared with the old subjects, in both the obese and normal weight groups (n=4 in each group). **C**, In keeping with the dihydroethidium (DHE) results, the concentration of malondialdehyde (MDA) increases with aging in the old obese compared with the young obese group (n=6 in each group). Data are presented as mean±SD. One-way ANOVA using the Tukey post hoc test for multiple comparisons and independent samples Student *t* test for pairwise comparisons were used to assess significant differences between groups. DAF-FM indicates 4-amino-5-methylamino-2',7'-difluorofluorescein.

by aging. In young obese, inhibition of arginase was able to significantly improve microvascular endothelial function, whereas this response was attenuated in the old obese group, despite the increased levels of vascular arginase I and II expression. This is likely related to the progressive increase of vascular oxidative stress observed with aging. Among the various source of vascular oxidative stress, we show that uncoupling of the eNOS because of elevated levels of arginase provides a minimal contribution to endothelial dysfunction in obese patients because of the hyperactivity of the NADPH oxidase. Although the concomitant neutralization of arginase and oxidative stress production induced a significant improvement of the microvascular function in young obese, old obese, and old controls, the maximal vasodilation obtained with these stimulations remained lower compared with the young control group and showed an inverse relationship with the amount of vascular remodeling. These findings suggest that the influence of arginase on endothelial-dependent microvascular relaxation in obesity is influenced by levels of vascular oxidative stress and is associated with severity of vascular remodeling. Our result support adoption of early treatments targeting arginase activity to restore the endothelial function in young obese subjects and to prevent the irreversible consequences of vascular remodeling.

A potential influence of arginase on endothelial function has been described in experimental models of obesity,[13–15] diabetes mellitus, hypertension,[27] and accelerated atherosclerosis.[28] Only 1 study provided evidence of a possible impact of arginase on endothelial function in obese patients with insulin resistance.[15] However, this effect was limited to obese patients with insulin resistance, and the authors did not explore the combined influence of oxidative stress and arginase inhibition on endothelial function at different ages. We now show that the influence of arginase on endothelial function in obesity is modified by aging, identifying NADPH oxidase activity as the most important pathway accounting for this regulation. Indeed, inhibition of NADPH oxidase can restore the endothelial response to norNOHA and further increase the ACh-dependent vasodilation obtained by norNOHA alone in the old obese group. We also clarify that, despite the greater amount of vascular arginase I and II in old age groups, production of superoxide production by an

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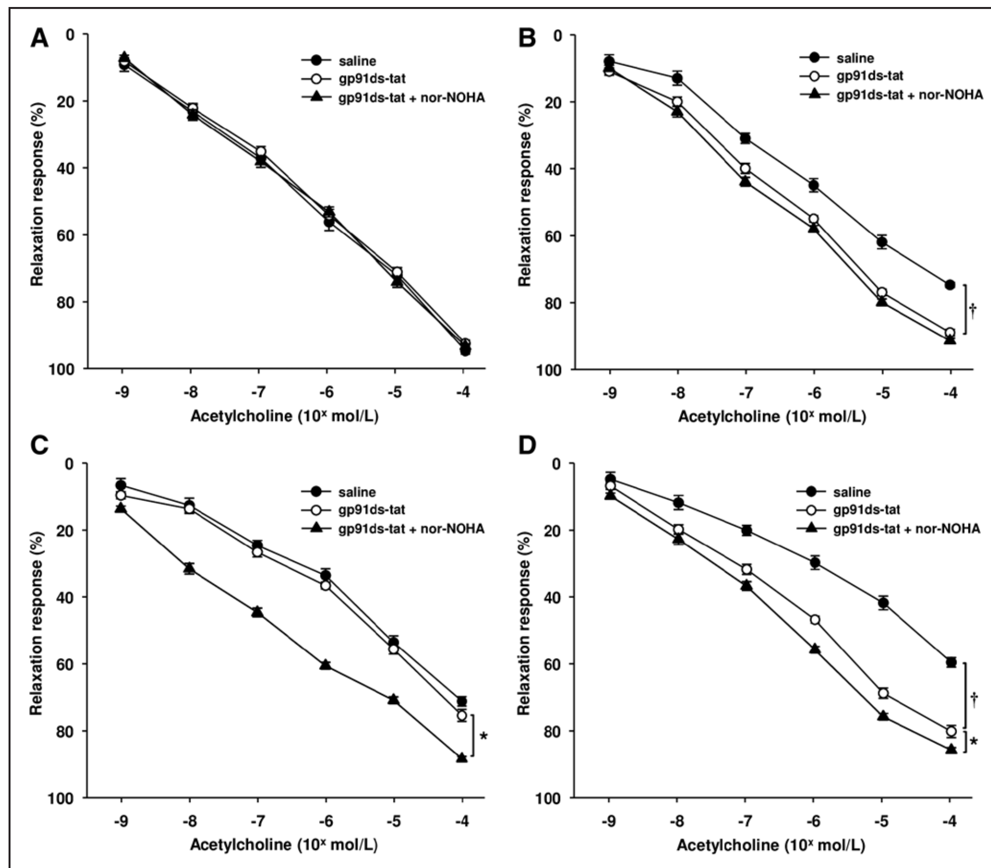

Figure 6. Improvement of endothelial function after inhibition of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase with gp91ds-tat. The greater improvement of endothelial function after addition of gp91ds-tat was observed in the older control and obese groups compared with younger obese. ANOVA for repeated measures, followed by the Student-Newman-Keuls test, was used to assess differences in the vasodilatory responses induced by preincubation with different stimuli. †$P<0.001$. *$P<0.05$. norNOHA indicates N(ω)-hydroxy-nor-L-arginine.

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uncoupled eNOS provides a minimal contribution to the endothelial dysfunction observed in old obese subjects. Our data highlight a discrepancy between the amount of vascular arginase and its influence on endothelial function. This is in keeping with previous evidence[29] and suggests that the simple assessment of arginase expression in the vascular wall does not provide a reliable estimate of its contribution to endothelial dysfunction in aging. Regulation of NO availability within endothelial cells is complex and dependents on the balance between its production and consumption. Availability of l-arginine is crucial to regulate eNOS activity. In physiological conditions, the high Km of arginase (>1 mmol/L) compared with eNOS (2–20 micromol/L) makes unlikely substrate competition as a limiting step in NO production.[30] However, overexpression of iNOS (inducible nitric oxide synthase) observed with aging and under proinflammatory conditions can cause S-nitrosylation of the cysteine residue C303 on arginase I, reducing its Km value 6-fold.[31] This might increase the ability of arginase I to influence endothelial function in diseases characterized by an elevated inflammatory exposure and during aging, improving its ability to compete with eNOS for l-arginine. These findings led to the hypothesis that inhibition of arginase (particularly the isoform I) might improve eNOS activity, potentially representing an important therapeutic target to improve age-related endothelial dysfunction.[32,33] However, no studies have previously explored the relationship between arginase activity and levels of oxidative stress at different ages. This is important as aging is also associated with a progressive increase of oxidative stress within the vascular wall.[34] An excess of free radicals rapidly consumes NO,[34] potentially nullifying the benefits of a higher NO production obtained with inhibition of arginase. Our results show that, while in physiological aging the elevated production of free radicals is likely to represent the most important factor regulating NO availability within the vascular wall, in obesity, the activity of arginase might provide an additional contribution, although this is likely to be attenuated, as in the control group, by the age-dependent increased activity of the NADPH oxidase. Thus, the functional results of our study suggest that the contribution of arginase to the obesity-related endothelial dysfunction is likely to be influenced by aging, which induces both an increased arginase and NADPH oxidase activities within the wall of small resistance vessels. As such, the influence of arginase on the endothelial dysfunction in age groups older than those included in our study could be different. Beyond the production of high levels of intracellular superoxide anions, this enzyme is also able to stimulate arginase expression.[35] Therefore, the increased activity of NADPH oxidase in old obese groups might explain both,

the reduced vasodilation obtained after arginase inhibition and the increased levels of arginase detected in the vascular wall of old age groups.

Another important finding of our study is the evidence of an accelerated process of microvascular remodeling in obese subjects, whom severity is related to the vascular wall levels of arginase I and II. Vascular aging is associated with changes in arterial wall structure and function, including luminal enlargement, vessel wall thickening because of intimal and medial expansion, collagen deposition, and impaired vasomotor function associated with endothelial dysfunction.[22,36–38] In our study, we provide evidence that obesity modifies the relationship between all these parameters and age. Although elevated oxidative stress in vessels from obese subjects could partially account for this accelerated process of vascular remodeling,[22] as suggested by the presence of a hypertrophic remodeling in older groups,[11] an additional contribution could be provided by the overexpression of arginase I and II in the vascular wall of obese and aged subjects. Arginase redirects the metabolism of L-arginine to L-ornithine, a substrate which is used by ornithine decarboxylase and aminotransferase for the formation of polyamines and L-proline, respectively. These, in turn, are essential for vascular smooth muscle cell proliferation[39] and collagen synthesis.[40] In addition, although the contribution of arginase to aging-related endothelial dysfunction might be covered by the increased production of oxidative stress, its influence on other pathways involved in endothelial cell aging might remain significant. Zhu et al[12] demonstrated that in cultured endothelial cells from umbilical veins higher levels of arginase I induce vascular endothelial inflammation by increasing expression of inflammatory VCAM-1 (vascular adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1), ultimately promoting monocyte adhesion to endothelial cells. Collectively, these arginase-driven events suggest that the elevated levels of arginase observed in our obese groups might contribute to the accelerated process of vascular aging observed in this condition. Importantly, the relaxing response obtained with coinhibition of NADPH oxidase and norNOHA remained lower in the obese and aged groups compared with young controls and was inversely related to the amount of microvascular remodeling. This suggests that, once the structural damage is established, blocking all functional pathways contributing to the obesity and age-related endothelial dysfunction fails to completely restore healthy vasodilation, the amount of which remains limited by the severity of the vascular remodeling.

Conclusions

In small human arteries, arginase contributes to endothelial dysfunction detected in obese subjects by reducing NO availability. The influence of arginase on microvascular endothelial function is attenuated by aging, as it is modulated by levels of vascular oxidative stress. However, the amount of arginase in the vascular wall is increased in obesity and aging and is directly related to the amount of vascular wall remodeling. Our findings suggest that early treatments targeting arginase activity can reduce microvascular endothelial dysfunction in obesity and prevent the vascular remodeling observed with aging and obesity.

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Disclosures

None.

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Highlights

- We show that arginase contributes to the endothelial dysfunction detected in small arteries of obese patients.
- Its influence on endothelial function is modulated by aging.
- This modulation is related to the high levels of vascular oxidative stress detected in old as compared with young healthy controls and young obese subjects.
- Expression of arginase in the microvascular wall is higher in obesity and aging and that its levels are related to parameters of microvascular remodeling, including media-lumen ratio, media cross-sectional area and amount of vascular wall collagen.

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