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ORIGINAL RESEARCH

Vascular Endothelial Function in Midlife/Older Adults Classified According to 2017 American College of Cardiology/American Heart Association Blood Pressure Guidelines

Daniel H. Craighead, PhD*; Kaitlin A. Freeberg, MS*; Douglas R. Seals, PhD

BACKGROUND: Impaired endothelial function is thought to contribute to the increased cardiovascular risk associated with above-normal blood pressure (BP). However, the association between endothelial function and BP classified by 2017 American College of Cardiology/American Heart Association guidelines is unknown. Our objective was to determine if endothelial function decreases in midlife/older adults across the 2017 American College of Cardiology/American Heart Association guidelines BP classifications and identify associated mechanisms of action.

METHODS AND RESULTS: A retrospective analysis of endothelial function (brachial artery flow-mediated dilation) from 988 midlife/older adults (aged 50+ years) stratified by BP status (normal BP; elevated BP; stage 1 hypertension; stage 2 hypertension) was performed. Endothelium-independent dilation (sublingual nitroglycerin), reactive oxygen species–mediated suppression of endothelial function (Δbrachial artery flow-mediated dilation with vitamin C infusion), and endothelial cell and plasma markers of oxidative stress and inflammation were assessed in subgroups. Compared with normal BP (n=411), brachial artery flow-mediated dilation was 12% (P=0.04), 15% (P<0.01) and 20% (P<0.01) lower with elevated BP (n=173), stage 1 hypertension (n=248) and stage 2 hypertension (n=156), respectively, whereas endothelium-independent dilation did not differ (P=0.14). Vitamin C infusion increased brachial artery flow-mediated dilation in those with above-normal BP (P≤0.02) but not normal BP (P=0.11). Endothelial cell $p47^{phox}$ (P<0.01), a marker of superoxide/reactive oxygen species–generating nicotinamide adenine dinucleotide phosphate oxidase, and circulating interleukin-6 concentrations (P=0.01) were higher in individuals with above-normal BP.

CONCLUSIONS: Vascular endothelial function is progressively impaired with increasing BP in otherwise healthy adults classified by 2017 American College of Cardiology/American Heart Association guidelines. Impaired endothelial function with above-normal BP is mediated by excessive reactive oxygen species signaling associated with increased endothelial expression of nicotinamide adenine dinucleotide phosphate oxidase and circulating interleukin-6.

Key Words: hypertension ■ inflammation ■ NADPH oxidase ■ oxidative stress

Above-normal resting (casual) blood pressure (BP), defined as systolic BP (SBP) ≥120 mm Hg or diastolic BP (DBP) ≥80 mm Hg, is associated with increased risk of cardiovascular disease (CVD), kidney disease, cognitive impairment, and other chronic disorders.[1–3] One of the nontraditional risk factors linking above-normal BP to these conditions is believed to be vascular endothelial dysfunction, most commonly characterized by impaired endothelium-dependent dilation.[4–7] Consistent with this notion, patients

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CLINICAL PERSPECTIVE

What Is New?

- This study assessed the relation between endothelial function and blood pressure (BP) in a large cohort of midlife and older adults classified according to the 2017 American College of Cardiology/American Heart Association BP guidelines.
- Compared with adults with normal BP, vascular endothelial function was progressively impaired in those with elevated BP and stage 1 and 2 hypertension.
- Multiple translational mechanistic approaches, including "pharmaco-dissection," endovascular biopsies of endothelial cells, and circulating biomarkers, show that the impaired endothelial function of groups with above-normal BP was associated with increased reactive oxygen species bioactivity, endothelial cell nicotinamide adenine dinucleotide phosphate oxidase $p47^{phox}$, and plasma interleukin-6.

What Are the Clinical Implications?

- Understanding the impact of above-normal BP based on current American College of Cardiology/American Heart Association guidelines, but below previous clinical cutoffs for hypertension, on vascular endothelial function in adults is important for optimal management of cardiovascular disease risk in the ever-growing population of midlife and older adults.
- New insight into the cellular and molecular mechanisms that may contribute to impaired endothelial function in groups with above-normal BP may help identify potential therapeutic targets for novel therapies.
- Our results support the need for healthy lifestyle-based strategies, in particular, to lower BP and improve endothelial function in midlife and older adults in all above-normal BP classifications according to the 2017 American College of Cardiology/American Heart Association guidelines.

Nonstandard Abbreviations and Acronyms

ACC	American College of Cardiology
AHA	American Heart Association
BMI	body mass index
CVD	cardiovascular disease
DBP	diastolic blood pressure
FMD_{BA}	brachial artery flow-mediated dilation
NADPH	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
SBP	systolic blood pressure

with hypertension, defined previously as SBP/DBP ≥140/90 mm Hg,[8,9] often demonstrate impaired endothelium-dependent dilation,[10,11] which, in turn, predicts incident risk of CVD.[1,2,12–14]

In 2017, the American College of Cardiology (ACC), American Heart Association (AHA), and other professional societies updated the Guidelines for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults.[15] This update created an "elevated" BP classification (SBP 120–129 and DBP <80 mm Hg) while revising downward the BP levels defining stage 1 (SBP 130–139 or DBP 80–89 mm Hg) and stage 2 (SBP ≥140 or DBP ≥90 mm Hg) hypertension.[15] These changes in BP guidelines were based on new epidemiologic and clinical information suggesting BP that is above normal, but below the previously defined threshold for hypertension (ie, SBP 120–139/DBP 80–89 mm Hg), is associated with increased cardiovascular risk and mortality.[16] On the basis of these revised guidelines, more than half of adults >50 years of age have hypertension, whereas less than a third have normal BP.[17]

Although understanding the inherent pathophysiological processes that underlie this recent reclassification is essential to develop optimal strategies for clinical BP management, no information presently is available related to these new guidelines. In this context, vascular endothelial dysfunction is a likely pathophysiological mechanism that contributes to the progressively greater cardiovascular risk of increasing BP across the 2017 ACC/AHA classifications. However, the relation between BP stratification in these current guidelines and endothelial function, as well as the potential underlying mechanisms, have not been established. Accordingly, the primary aim of this study was to determine, among otherwise healthy midlife/older adults with no known presence of other chronic clinical diseases, if endothelial function decreases with increasing BP status based on the 2017 ACC/AHA guidelines. If so, our secondary aim was to gain initial insight into the potential clinical factors and physiological mechanisms through which endothelial function is associated with BP status per these new guidelines.

To address our primary aim, we first determined the association between brachial artery flow-mediated dilation (FMD_{BA}), a well-established measure of endothelium-dependent dilation and vascular endothelial function, and BP across the 2017 ACC/AHA BP classifications in a large sample of adults ≥50 years of age from our laboratory database. To address our secondary aim, we then determined if differences in other clinical characteristics, such as age or blood lipids, explained, at least in part, BP-based group differences in endothelial function. Because antihypertensive medications influence vascular endothelial

function,[18,19] we also assessed whether BP-related group differences in endothelial function were apparent when examining only unmedicated individuals. Next, we determined if differences in vascular smooth muscle sensitivity to NO, that is, endothelium-independent dilation, might explain group differences in endothelial function (FMD_{BA}). Finally, we determined if reactive oxygen species (ROS)-associated suppression of endothelial function (ie, change in FMD_{BA} in response to acute supratherapeutic infusion of the antioxidant ascorbic acid [vitamin C]) or endothelial cell or circulating markers of oxidative stress and inflammation were linked to differences in endothelial function across BP classifications.

METHODS

All procedures were reviewed and approved by the Institutional Review Board at the University of Colorado Boulder. The nature, benefits, and risks of all study procedures were explained to volunteers, and their written informed consent was obtained before study participation. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Subjects

A retrospective analysis of data collected on 988 midlife/older adults (50–79 years) who underwent testing for casual (resting) BP and vascular endothelial function by our laboratory with well-standardized procedures at the University of Colorado Boulder between 2008 and 2019 was performed. Subjects were included if they were in the appropriate age range and had undergone standardized measurements of casual BP, vascular endothelial function, and clinical characteristics. Individuals from all racial, ethnic, and socioeconomic backgrounds were included, but the majority of subjects were White and of higher education and income status. No subjects were current smokers, and all subjects were free of overt clinical disease, including CVD, based on a medical history, physical examination, resting and maximal exercise BP and ECG, and standard blood chemistries.[20–24] Individuals taking prescription antihypertensive medications and other classes of prescription medications (eg, statins) were included provided their drug regimen was stable for the previous ≥3 months. Subjects were separated into 4 groups according to 2017 ACC/AHA guidelines: normal BP (SBP <120 and DBP <80 mm Hg; n=411); elevated BP (SBP 120–129 and DBP <80 mm Hg; n=173); stage 1 hypertension (SBP 130–139 or DBP 80–89 mm Hg; n=248); and stage 2 hypertension (SBP ≥140 or DBP ≥90 mm Hg; n=156).

Measurements

All measurements were taken following an overnight fast and abstaining from caffeine for 12 hours, and alcohol and vigorous exercise for 24 hours. Casual BP was determined as seated brachial artery BP measured in triplicate after ≥5 minutes of quiet rest according to World Health Organization, Joint National Committee 7, and ACC/AHA guidelines.[8,9,15] Measurements were either taken manually by a trained investigator via brachial auscultation or by an automated oscillometric sphygmomanometer. These 2 methods have been shown to yield similar BP measurements.[25,26] The average of the 3 BP measurements was taken and used for group allocation.

Endothelium-Dependent Dilation

FMD_{BA} was assessed using high-resolution ultrasonography in response to a 5-minute period of blood flow occlusion with a cuff positioned on the upper forearm, as described previously by our laboratory[27–30] and others.[31,32] For more detail, see Data S1. FMD_{BA} was quantified as percentage change from baseline diameter, calculated as $FMD_{BA}(\Delta\%)=((\text{Peak Diameter}-\text{Baseline Diameter})/\text{Baseline Diameter})\times 100$.[31,33,34]

Endothelium-Independent Dilation

A subset of subjects underwent testing for endothelium-independent dilation (n=259), that is, brachial artery dilation in response to 0.4 mg of sublingual nitroglycerin, to assess vascular smooth muscle sensitivity to NO.[27,28,35] Ultrasound images of the brachial artery were captured at baseline and for 10 minutes following administration. The maximal brachial artery dilation to nitroglycerin was calculated as a percentage change in diameter from baseline.[31,33,34]

ROS-Associated Suppression of Endothelium-Dependent Dilation

To gain causal mechanistic insight, tonic suppression of endothelial function by ROS was assessed in a subset of subjects (n=234) using a supratherapeutic systemic infusion of vitamin C (ascorbic acid), as described previously by our laboratory.[27,36] The change in FMD_{BA} from baseline (preinfusion) in response to vitamin C was interpreted as the magnitude of tonic ROS-associated suppression of endothelium-dependent dilation (vascular endothelial function).[27,36] Additional details are available in Data S1.

Endothelial Cell Analysis via Endovascular Biopsy

The abundance of protein markers of oxidative stress and inflammation was assessed in biopsied endothelial cells collected on J-wires advanced into an

Table. Subject Characteristics

	Normal N=411	Elevated N=173	Stage 1 N=248	Stage 2 N=156
SBP, mm Hg	108±8	124±3*	131±7*	147±9*
DBP, mm Hg	67±6	72±5*	79±6*	85±9*
Age, y	62±6	65±7*	64±7*	64±7*
Women, %	62	49*	38*	47*
BMI, kg/m^2	24.1±3.9	25.1±3.9*	26.2±3.9*	26.5±4.1*†
Triglycerides, mg/dL	94±50	104±52	115±58*	113±58*
HDL-C, mg/dL	63±18	57±16*	57±18*	57±18*
LDL-C, mg/dL	118±30	114±27	121±29	119±33
Glucose, mg/dL	88±9	89±8	90±10*	89±11
BP medication, %	3.2	9.8*	12.6*	15.6*
Other prescription medications, %	28.9	32.9	32.9	38.7

Continuous variables are means±SD. Categorical variables are percentages. BMI indicates body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and SBP, systolic blood pressure.

*P<0.05 compared with normal BP.

†P<0.05 compared with elevated BP.

‡P<0.05 compared with stage 1 hypertension.

antecubital vein via immunofluorescent staining, as previously described[29,37,38] and as presented in detail in Data S1. Our laboratory established previously that protein markers assessed in venous endothelial cells correlate well with the same markers assessed in arterial endothelial cells obtained by invasive brachial artery catheterization.[29,37] For analysis, endothelial cells were stained with primary antibodies for nitrotyrosine (posttranslational oxidative modification; n=240), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p47^{phox} (pro-oxidant enzyme marker; n=187), manganese superoxide dismutase (antioxidant enzyme; n=96), and nuclear factor kappa B p65 (proinflammatory transcription factor; n=197), as well as with an AlexaFluor fluorescent secondary antibody (Invitrogen, Carlsbad, CA). Cells were viewed with a fluorescence microscope and analyzed with Metamorph Software. Endothelial cells were identified by cell staining for von Willebrand factor and nuclear integrity was confirmed via 4′,6′-diamidino-2-phenylindole hydrochloride staining.[29,30,37] Values are expressed as ratios of endothelial cell protein expression to human umbilical vein endothelial cells to minimize the effects of differences in staining intensities across multiple staining sessions.[29,30,37]

Markers of Systemic Oxidative Stress and Inflammation

Circulating plasma markers of inflammation (interleukin-6, n=331; tumor necrosis factor alpha, n=225; high-sensitivity C-reactive protein, n=432), oxidative stress (oxidized low-density lipoprotein [LDL], n=452), and antioxidant defenses (total antioxidant status, n=278) were analyzed using ELISA (interleukin-6; tumor necrosis factor alpha; oxidized LDL), immunoturbidimetry (high-sensitivity C-reactive protein), and the oxidative method (total antioxidant status).[22]

Statistical Analysis

Statistical comparisons were made in SPSS version 25 using the General Linear Model. Sidak post hoc tests were used to assess between-group mean differences for continuous variables. Linear regression was used to determine how endothelial function was related to ACC/AHA BP classifications, ROS-associated suppression of endothelial function, endothelial cell NADPH oxidase p47^{phox}, and circulating interleukin-6 concentration. Multiple linear regression was used to account for additional clinical characteristics (eg, age) explaining differences in endothelial function. The Mixed Linear Model was used to determine group by condition (saline versus vitamin C) interaction for ROS-associated suppression of FMD_{BA}; the Sidak post hoc test was used to assess the within-group effect of vitamin C. Because of the smaller sample sizes available as a result of the highly technical nature of measurements, for the endothelial cell markers of inflammation and oxidative stress, subjects were classified as having either normal (SBP <120 mm Hg and DBP <80 mm Hg) or above-normal BP (SBP ≥120 mm Hg or DBP ≥80 mm Hg) based on the 2017 ACC/AHA guidelines, and group comparisons were made with the General Linear Model. Subject characteristics for each subset analysis presented in the results were not different from the characteristics of the whole group. Categorical variables were compared with the chi-square test. Unless otherwise specified, continuous variables in the text and

table are expressed as mean±SD while categorical variables are expressed as percentages. Statistical significance was set a priori at α=0.05.

RESULTS

Clinical Characteristics

Clinical characteristics are presented in Table. By design, BP differed between all groups, with SBP and DBP increasing across ACC/AHA BP classifications. Excluding BP status in those with above-normal BP, subjects were free of clinical metabolic or cardiovascular diseases. Although selective group differences existed, mean values were all within clinical norms. All groups with above-normal BP were 2 to 3 years older (P<0.01), had a slightly higher body mass index (BMI) (P≤0.02) and modestly lower high-density lipoprotein cholesterol (P<0.01), were more likely to be men (P<0.05), and were more likely to be taking antihypertensive medications (P<0.05) than subjects with normal BP. Those with stage 1 hypertension had higher fasting glucose than normotensive subjects (P=0.01). Subjects with stage 1 or stage 2 hypertension had higher triglycerides than normotensive subjects (P<0.01). Finally, those with stage 1 and stage 2 hypertension also had a modestly higher BMI than the group with elevated BP (P<0.03).

Endothelial Function

Overall Cohort

On the basis of FMD_{BA}, endothelial function was significantly impaired in midlife/older adults with elevated BP (5.2±2.6Δ%, P=0.04), stage 1 hypertension (5.1±2.7Δ%, P<0.01), and stage 2 hypertension (4.7±2.4Δ%, P<0.01) compared with midlife/older adults with normal BP (5.9±2.9Δ%) (Figure 1). Although there was no significant difference in mean FMD_{BA} among the 3 groups with above-normal BP, there was a significant inverse relation between FMD_{BA} and increasing BP status (elevated BP, –12%; stage 1 hypertension, –15%; stage 2 hypertension, –20% versus normal BP group; r=–0.157; P<0.01).

Influence of Clinical Factors

BP classification, along with age, sex, BMI, serum triglycerides, high-density lipoprotein cholesterol, LDL cholesterol, and fasting blood glucose were entered into the linear model. Fit for the whole model was R^2=0.05. Within the model, BP classification was related to FMD_{BA} (β=–0.325; 95% CI, –0.481 to –0.161; P<0.01; partial R^2=0.02), such that those individuals in the above-normal BP classifications were more likely to have lower values of FMD_{BA}. Age was also related to FMD_{BA} (β=–0.051; 95% CI, –0.077 to –0.026; P<0.01; partial R^2=0.016), such that being of older age was associated with lower values of FMD_{BA}. Finally, sex was related to FMD_{BA} (β=–0.420; 95% CI, –0.796 to –0.043; P=0.03; partial R^2=0.005) with men tending to have lower values of endothelial function than women. The other variables in the model, including BMI (P=0.95), triglycerides (P=0.53), high-density lipoprotein cholesterol (P=0.92), LDL cholesterol (P=0.56), and fasting glucose (P=0.66), were not significantly related to FMD_{BA}.

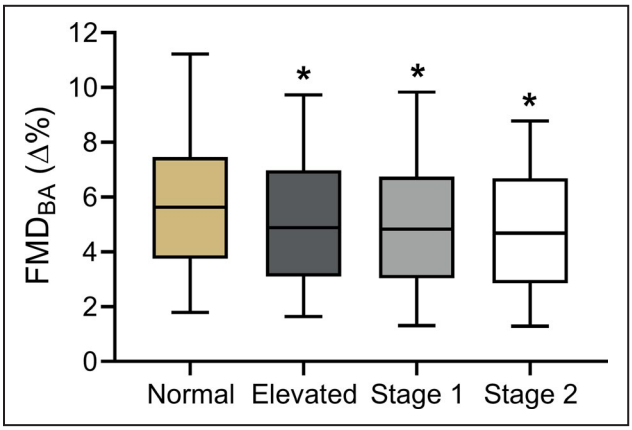

Figure 1. Vascular endothelial function (endothelium-dependent dilation).
Brachial artery flow-mediated dilation (FMD_{BA}) in midlife/older adults classified according to 2017 American College of Cardiology/American Heart Association blood pressure (BP) guidelines. Box indicates interquartile range and median, whiskers indicate 5th to 95th percentiles. Normal BP: n=411; elevated BP: n=173; stage 1 hypertension: n=248; stage 2 hypertension: n=156. *P<0.05 compared with normal BP.

Nonmedicated Individuals

FMD_{BA} also was determined in the subgroup of the overall cohort who were not prescribed BP-lowering medications to assess the influence of BP status on endothelial function independent of the effects of antihypertensive pharmacotherapy. Compared with those with normal BP (n=398; FMD_{BA} 5.9±2.9Δ%), FMD_{BA} tended to be lower in adults with elevated BP (n=156; FMD_{BA} 5.3±2.6Δ%; P=0.09) and was significantly lower in adults with stage 1 hypertension (n=217; FMD_{BA} 5.1±2.7Δ%; P<0.01) or stage 2 hypertension (n=132; FMD_{BA} 4.8±2.3Δ%; P<0.01). Thus, the associations between FMD_{BA} and BP across the 2017 ACC/AHA BP classifications were similar in the overall cohort and in the subgroup not taking antihypertensive medications.

Endothelium-Independent Dilation

Endothelium-independent dilation, that is, vascular smooth muscle sensitivity to NO, was assessed by brachial artery dilation in response to sublingual nitroglycerin in a subset of subjects within the overall cohort. As in the overall cohort, FMD_{BA} was lower in

the subgroups with above-normal BP versus the subgroup with normal BP (range, –15% to –21%). Brachial artery dilation in response to sublingual nitroglycerin did not differ across the normal (24.6±7.1Δ%), elevated BP (22.5±4.7Δ%), stage 1 hypertension (23.0±7.2Δ%) and stage 2 hypertension (25.0±6.2Δ%) subgroups (P=0.14), indicating no BP status-related differences in vascular smooth muscle sensitivity to NO (Figure 2).

Association With Oxidative Stress and Inflammation

ROS-Associated Suppression of Endothelial Function

In subjects in whom supratherapeutic concentrations of the antioxidant vitamin C were infused to determine tonic ROS-associated suppression of endothelium-dependent dilation, vitamin C significantly increased FMD_{BA} above baseline (saline control) in those with elevated BP (saline, 4.6±2.4Δ%; vitamin C, 5.5±2.3Δ%; P=0.02), stage 1 hypertension (saline, 5.0±2.6Δ%; vitamin C, 5.7±3.0Δ%; P=0.01), and stage 2 hypertension (saline; 4.6±2.2Δ%; vitamin C, 5.6±2.7Δ%; P<0.01), but not in those with normal BP (saline, 5.4±3.0Δ%, vitamin C, 5.8±3.1Δ%; P=0.12) (Figure 3). There was a modest but statistically significant inverse correlation between the change in FMD_{BA} with vitamin C infusion and baseline FMD_{BA} (r=–0.14; P=0.04).

Molecular Characteristics of Endothelial Cells Obtained via Endovascular Biopsy

To assess potential molecular mechanisms underlying impairments in vascular endothelial function in the groups with above-normal compared with normal BP, the abundance of protein markers associated with oxidative stress and inflammation was measured in endothelial cells obtained from subsets of subjects via endovascular biopsy (Figure 4). Expression of $p47^{phox}$, a subunit of selective isoforms of the superoxide/ROS-generating enzyme NADPH oxidase, was higher in the groups with above-normal BP (0.75±0.44 AU) compared with the group with normal BP (0.55±0.26 AU; P<0.01). The abundance of nitrotyrosine (normal, 0.69±0.68 AU; above-normal, 0.67±0.56 AU; P=0.78), manganese superoxide dismutase (normal, 0.49±0.26 AU; above-normal, 0.48±0.36 AU; P=0.89), and manganese superoxide dismutase (normal, 0.39±0.35 AU; above-normal, 0.44±0.31 AU; P=0.37) were not different between the groups. There were no significant relations between any endothelial cell marker and FMD_{BA} (all P>0.05).

Circulating Markers of Systemic Oxidative Stress and Inflammation

To gain insight into whether systemic inflammation and oxidative stress differed between groups with normal and above-normal BP, circulating plasma markers of inflammation, oxidative stress, and antioxidant defenses were measured in subsets of subjects (Figure 5). Interleukin-6, a proinflammatory cytokine, was higher in those with above-normal BP (1.81±2.54 pg/mL) compared with normotensive subjects (1.25±1.16 pg/mL; P=0.01), and there was a modest but statistically

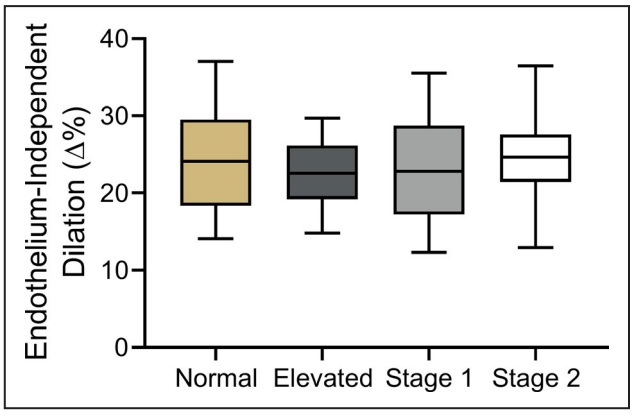

Figure 2. Endothelium-independent dilation.
Brachial artery dilation in response to sublingual nitroglycerin in midlife/older adults classified according to 2017 American College of Cardiology/American Heart Association guidelines. Box indicates interquartile range and median, whiskers indicate 5th to 95th percentiles. Normal blood pressure (BP): n=96; elevated BP: n=42; stage 1 hypertension: n=84; stage 2 hypertension: n=37.

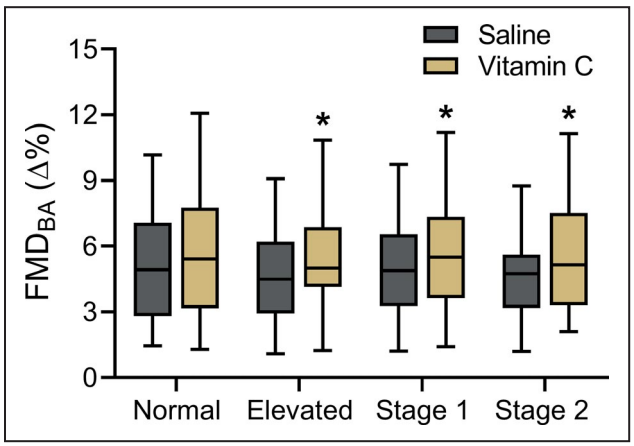

Figure 3. Tonic reactive oxygen species-mediated suppression of endothelial function.
Brachial artery flow-mediated dilation (FMD_{BA}) following intravenous infusion of saline and vitamin C in midlife/older adults classified according to 2017 American College of Cardiology/American Heart Association guidelines. Data are expressed as percent change (Δ%) from baseline diameter. Box indicates interquartile range and median, whiskers indicate 5th to 95th percentiles. Normal blood pressure (BP): n=100; elevated BP: n=34; stage 1 hypertension: n=64; stage 2 hypertension: n=36. *P<0.05 compared with saline condition.

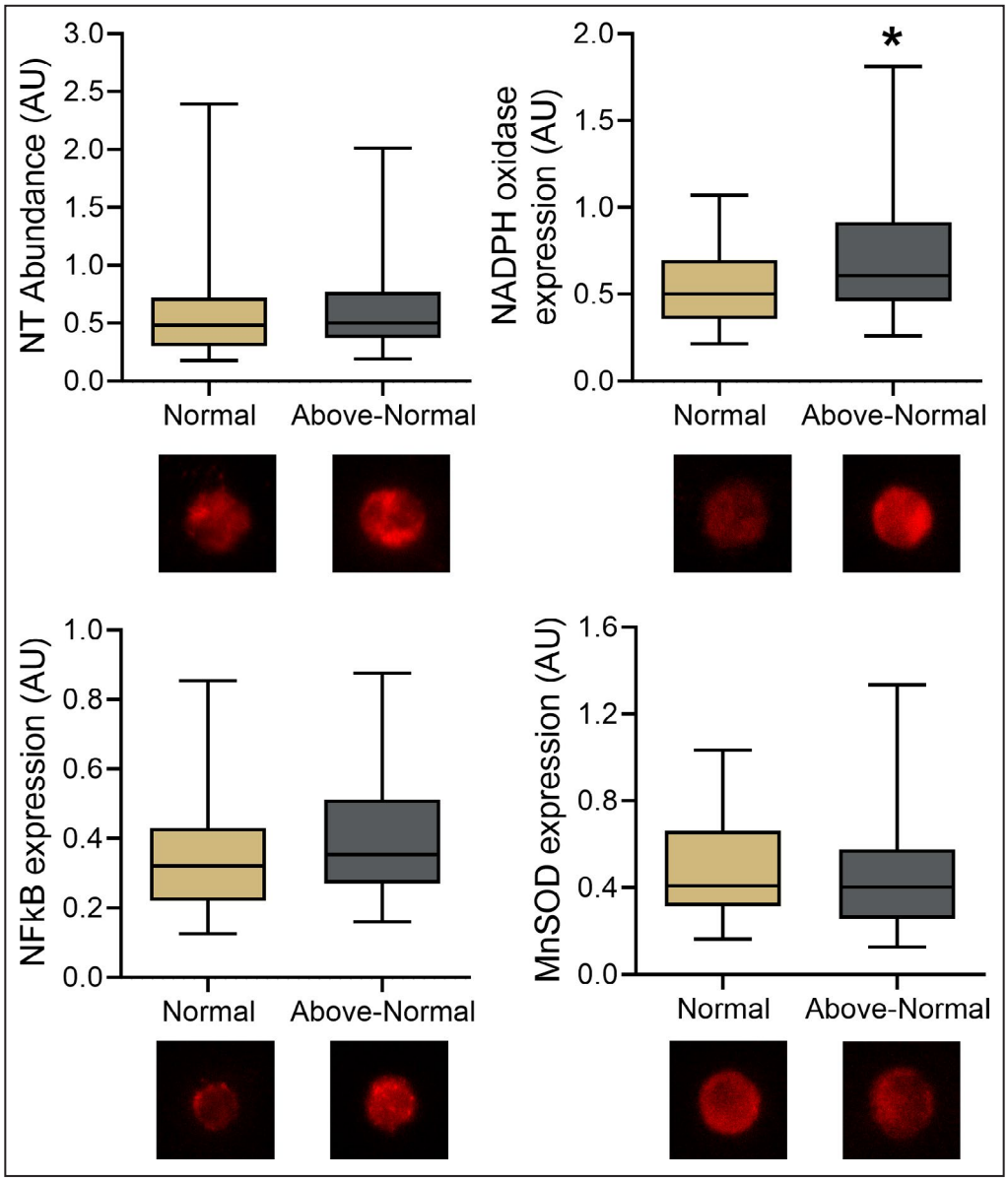

Figure 4. Endothelial cell protein markers of oxidative stress and inflammation.
Abundance of nitrotyrosine (NT; oxidative modification of proteins) and expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (oxidant-producing enzyme), manganese superoxide dismutase (MnSOD; antioxidant defenses) and nuclear factor κ B (NFκB; proinflammatory transcription factor) in biopsied venous endothelial cells from midlife/older adults with normal blood pressure (BP; <120/80 mm Hg systolic BP [SBP]/diastolic BP [DBP]) vs above-normal BP (≥120 mm Hg SBP or ≥80 mm Hg DBP) with example immunofluorescent images of individual endothelial cells below. Data are normalized to human umbilical vein endothelial cell protein expression via immunofluorescence. Box indicates interquartile range and median, whiskers indicate 5th to 95th percentiles. *P<0.05 compared with normal BP.

significant inverse relation between interleukin-6 and FMD_{BA} (r=−0.14; P=0.01; n=331). There were no significant differences between groups in plasma oxidized LDL (normal, 56.49±18.77 U/L; above-normal, 58.50±18.55 U/L; P=0.29), total antioxidant status (normal, 1.25±0.21 mmol/L; above-normal, 1.30±0.22 mmol/L; P=0.06), high-sensitivity C-reactive protein (normal, 1.08±1.29 mg/L; above-normal, 1.20±1.57 mg/L; P=0.41), or tumor necrosis factor alpha (normal, 1.41±1.25 pg/L; above-normal, 1.51±1.27 pg/L; P=0.47).

DISCUSSION

In this investigation, we assessed the relation between vascular endothelial function and BP status in almost 1000 men and women ≥50 years of age.

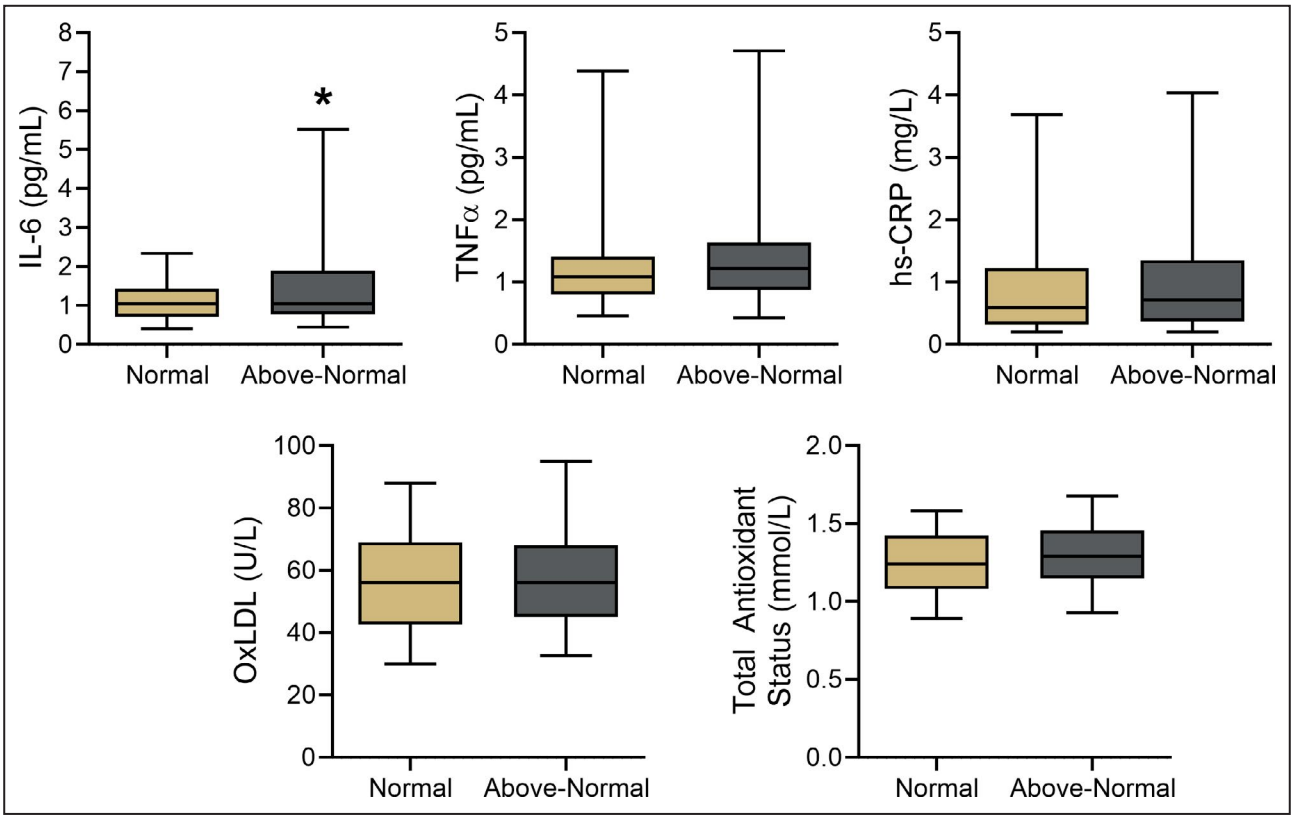

Figure 5. Circulating concentrations of inflammatory, oxidative stress and antioxidant markers.
Plasma concentrations of inflammatory (IL-6 [interleukin-6]; TNFα [tumor necrosis factor alpha]; hsCRP [high-sensitivity C-reactive protein]), oxidative stress (oxidized LDL [OxLDL]), and antioxidant (total antioxidant status) markers in midlife/older adults with normal blood pressure (BP; <120/80 mm Hg systolic BP [SBP]/diastolic BP [DBP]) vs above-normal BP (≥120 mm Hg SBP or ≥80 mm Hg DBP). Box indicates interquartile range and median, whiskers indicate 5th to 95th percentiles. *P<0.05 compared with normal BP.

Our primary finding was that vascular endothelial function, assessed using FMD_{BA}, is progressively impaired in otherwise healthy midlife/older adults with elevated BP, stage 1 hypertension and stage 2 hypertension compared with their counterparts with normal BP based on the 2017 ACC/AHA guidelines.[15] At the extremes, FMD_{BA} was 20% lower in subjects with stage 2 hypertension compared with individuals with normal BP. Importantly, after controlling for other clinical characteristics (eg, age, BMI), BP classification remained independently related to FMD_{BA}. The lower FMD_{BA} in individuals in the above-normal BP classifications was not related to reduced smooth muscle sensitivity to NO, as brachial artery dilation in response to sublingual nitroglycerin did not differ across groups on the basis of BP classification, indicating an impairment in the vascular endothelium per se. Administration of the ROS-scavenging antioxidant molecule vitamin C improved FMD_{BA} only in individuals in the above-normal BP groups, suggesting that the impaired endothelial function in these groups was mediated by excessive ROS-associated suppression of endothelium-dependent dilation. This ROS-mediated inhibition of FMD_{BA} in individuals in the above-normal BP classifications was, in turn, associated with evidence of greater abundance of the ROS (superoxide)-producing enzyme, NADPH oxidase, in biopsied vascular endothelial cells. Finally, individuals in the above-normal BP groups demonstrated higher circulating concentrations of the proinflammatory marker interleukin-6 than their peers with normal BP, and interleukin-6 was inversely related to FMD_{BA} in the overall cohort.

Potential Clinical Implications

In the present analysis, there was a statistically significant trend for progressively lower FMD_{BA} among individuals classified with elevated BP, stage 1 hypertension and stage 2 hypertension, respectively. Meta-analyses show an inverse relation between FMD_{BA} and future CVD events, with a 1% (Δ% units) decrease in FMD_{BA} associated with an ≈8% to 13% increase in risk of future CVD events.[39–43] Compared with individuals with normal BP, we found that FMD_{BA} was 0.7 to 1.2 Δ% units lower in the groups with above-normal BP. These results suggest that the magnitude of the impairments in FMD_{BA} observed

in midlife/older adults in the above-normal BP classifications of the 2017 ACC/AHA guidelines may be clinically significant.

Other than the higher SBP of those individuals in the above-normal BP groups, the midlife/older adults included in this analysis were healthy. On the one hand, our cohort affords the unique experimental advantage of isolating, as much as is possible in free-living humans, the effects of differing BP status per se on endothelial function. On the other hand, it is possible, perhaps likely, that our results *underestimate* differences in endothelial function in the broader population of adults differing in BP status, particularly those with greater CVD risk factor burden or of lower socioeconomic status.

It may be noteworthy that the midlife/older adults in our analysis with BP in the "elevated" and "stage 1 hypertension" classifications based on the 2017 ACC/AHA BP cutoffs would not have been recommended for treatment under previous guidelines, despite having reduced FMD_{BA} (ie, impaired endothelial function). In this context, we and others have shown that healthy lifestyle-based interventions, including those recommended in ACC/AHA guidelines (eg, exercise, low-salt diet, weight loss), improve endothelial function in midlife/older adults with BP in the current elevated and stage 1 hypertension classifications.[20,44,45] As such, the present findings support the concept that evidence-based healthy lifestyle strategies should be encouraged across all stages of above-normal BP status, not only for their antihypertensive effects but also for their ability to improve vascular endothelial function.

Role of Clinical Factors

As anticipated, age and sex were also related to vascular endothelial function (FMD_{BA}). However, BP classification remained significantly related to FMD_{BA} after accounting for these and other clinical characteristics. Moreover, our results were largely unchanged when examining only those subjects not prescribed antihypertensive medications. As such, our findings suggest that having above-normal BP per se according to the 2017 ACC/AHA guidelines is associated with impaired endothelial function in midlife/older men and women without other chronic clinical disorders.

Vascular Smooth Muscle Sensitivity to NO

Our assessments of endothelium-independent dilation (brachial artery dilation in response to sublingual nitroglycerin) indicate that vascular smooth muscle sensitivity to NO does not differ among midlife/older adults across the 2017 ACC/AHA BP classifications (Figure 2). These findings indicate that group differences in FMD_{BA} are not explained by this mechanism but rather likely reflect impaired endothelial NO production or bioavailability, consistent with the results of previous reports based on smaller cohorts than the present study.[11,20,46] It is possible, however, that decreased responsiveness to NO contributes to impaired FMD_{BA} in midlife/older adults with a more adverse CVD risk factor profile than the healthier individuals included in our analysis.[5] Indeed, impaired endothelium-independent dilation has been reported in subjects with above-normal BP *and* increased adiposity,[47] chronic kidney disease,[48] and coronary heart disease.[49]

ROS-Related Suppression of Endothelial Function

Excess ROS, particularly superoxide, can impair endothelium-dependent dilation (FMD_{BA}) via direct reaction with NO and by inhibiting NO production by the enzyme endothelial NO synthase.[5,50] Vitamin C (ascorbic acid) infusion is a well-established model for gaining causal insight into the role of tonic ROS-related suppression of endothelial function in humans.[27,36,51,52] With this approach, vitamin C, a potent antioxidant, is infused to attain supratherapeutic/physiological circulating levels to temporarily (reversibly) reduce ROS bioactivity. The increase in endothelium-dependent dilation in response to vitamin C infusion is interpreted as reflecting the degree of tonic ROS-dependent suppression of vascular endothelial function.[27,36,51,52] In our analysis, intravenous infusion of vitamin C increased FMD_{BA} in individuals in the above-normal BP classifications but not in those with normal BP (Figure 3). Consistent with this observation, among individuals, baseline FMD_{BA} and the degree of tonic ROS-associated suppression of FMD_{BA} were inversely related. These experimental findings provide direct evidence suggesting that increased ROS bioactivity contributes to impaired vascular endothelial function in midlife/older adults with above-normal BP based on the 2017 ACC/AHA guidelines.

Endothelial Molecular Mechanisms

To gain insight into potential molecular mechanisms underlying ROS-mediated suppression of FMD_{BA} in individuals classified with above-normal BP, we analyzed data obtained from endovascular biopsies of endothelial cells in a subset of our subjects. We found that expression of the $p47^{phox}$ subunit of NADPH oxidase, a major superoxide/ROS-producing enzyme in the vasculature,[53,54] was higher in endothelial cells from individuals with above-normal versus normal BP (Figure 4). This finding suggests that increased expression of NADPH oxidase may have contributed to increased ROS bioactivity and its tonic suppression

of NO-mediated endothelial function in adults with above-normal BP. Of note, manganese superoxide dismutase, the mitochondrial isoform of superoxide dismutase, an important endogenous antioxidant enzyme for regulating superoxide/ROS in arteries,[55,56] was similar in individuals with normal and above-normal BP (Figure 4). This observation suggests a lack of an appropriate compensatory increase in endogenous antioxidant defenses in the face of increased superoxide/ROS bioactivity in the above-normal BP groups. Finally, nitrotyrosine, a posttranslational marker of oxidant modification of tyrosine residues on proteins, did not differ between the normal and above-normal BP groups. This may indicate that altered ROS signaling in individuals with above-normal BP, although functionally significant, did not attain levels necessary to induce a measurable increase in this indirect marker of oxidative stress.

Circulating Markers of Oxidative Stress and Inflammation

We found no differences in plasma oxidized LDL or total antioxidant status, indirect markers of systemic oxidative stress and antioxidant defenses, respectively, across the normal and above-normal BP classifications. Although circulating concentrations of high-sensitivity C-reactive protein and tumor necrosis factor alpha also did not differ among the groups, plasma interleukin-6, a major proinflammatory cytokine, was 46% greater in subjects with above-normal compared with normal BP (Figure 5) and was inversely related to FMD_{BA} among individuals in the overall cohort. By increasing angiotensin type 1 receptor expression, interleukin-6 stimulates production of ROS from vascular smooth muscle cells and induces endothelial dysfunction in mice.[57–59] Circulating concentrations of interleukin-6 also correlate inversely with FMD_{BA} and other measures of endothelial dysfunction among healthy midlife/older men[60] and participants of the Framingham Offspring Study,[61] which is positively associated with future cardiovascular events.[62,63] Thus, the greater circulating interleukin-6 in individuals with above-normal BP may have contributed to their lower FMD_{BA} in the present study.

Finally, we wish to emphasize that there are well-established links in hypertension between NADPH oxidase-stimulated superoxide production and systemic inflammatory signaling, as indicated by elevated levels of interleukin-6. For example, NADPH oxidase-associated oxidative stress is observed in antigen-presenting cells of hypertensive animals and humans and can stimulate release of interleukin-6 from mononuclear cells.[64,65] These events could, in turn, contribute to an imbalance in pro- versus anti-inflammatory T-cell regulation, thus linking NADPH oxidase activity to oxidative stress, inflammation, and endothelial dysfunction in the setting of hypertension.

Study Limitations

Mechanistic outcomes were measured in different subsets of the overall cohort, including subgroups of subjects who underwent the technically demanding vascular endothelial cell analyses via endovascular biopsy. Given the retrospective design of this analysis, it was not possible to assess all mechanistic outcomes in the same subjects as not all measurements were included in each prior investigation. When combined with inherent measurement variability, this limitation likely masked group differences in some protein markers or interindividual relations between those markers, such as $p47^{phox}$ abundance and FMD_{BA}. Moreover, because of the cross-sectional nature of our analysis, we cannot determine whether changes in endothelial function, oxidative stress, and inflammation are a cause or consequence of above-normal BP. Multiple investigators collected the vascular function data in this analysis, which may have increased variability; however, our laboratory used standardized data collection and analysis procedures for all of the measurements, which minimized variability as much as is possible. Finally, because of the small number of subjects prescribed antihypertensive medications, we were not able to perform direct comparisons of those receiving and not receiving BP medication.

CONCLUSIONS

In summary, our findings indicate that vascular endothelial function, as assessed by FMD_{BA}, is progressively impaired in otherwise healthy midlife/older adults with elevated BP, stage 1 hypertension, or stage 2 hypertension based on 2017 ACC/AHA guidelines compared with their peers with normal BP. We present evidence that the lower FMD_{BA} in individuals with above-normal BP may be mediated by tonic ROS-related suppression of endothelium-dependent dilation associated with increased endothelial cell expression of the superoxide-generating enzyme, NADPH oxidase. Higher circulating concentrations of the proinflammatory cytokine interleukin-6 also may contribute to impaired endothelial function in the subjects with above-normal BP.

Our results provide evidence that impaired endothelial function is apparent in midlife/older adults with above-normal BP per current ACC/AHA guidelines in the absence of other cardiometabolic clinical disorders. As such, differences in endothelial function may constitute an important pathophysiological “substrate” contributing to the increased risk of CVD across these recent BP reclassifications. Overall, our findings

support the implementation of healthy lifestyle practices and, possibly, pharmacological strategies to improve vascular endothelial function in individuals with BP in the elevated and stage 1 and 2 hypertension ranges based on the 2017 ACC/AHA guidelines.

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Disclosures

None.

Supplementary Materials

Data S1
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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Endothelium-dependent dilation. Endothelium-dependent dilation was assessed as brachial artery flow-mediated dilation (FMD_{BA}) using an ultrasound machine. The right arm was adducted at heart level and the brachial artery was located 3-6 cm above the antecubital crease. The ultrasound probe was then clamped to improve stability and avoid movement. After obtaining baseline diameters, reactive hyperemia was produced by inflating a blood pressure cuff placed on the upper forearm to 250 mmHg for 5 minutes. After 5 minutes the cuff was rapidly deflated. Brachial artery diameter and blood velocity were measure during the first 2 minutes post-occlusion to obtain the peak dilatory response. FMD_{BA} was calculated as the percentage change in brachial artery diameter in response to the forearm hyperemic stimulus.

Reactive oxygen species-associated suppression of endothelium-dependent dilation. The infusion of ascorbic acid (vitamin C) at supra-physiological concentrations temporarily reduces superoxide/reactive oxygen species (ROS) bioactivity, thus removing the “tonic” influence of excessive ROS. The acute increase (or lack thereof) in “function” (FMD_{BA}) is a measure of the tonic influence of the ROS under normal conditions. If function improves from baseline control levels, then there is tonic suppression by ROS; if function does not change, then the interpretation is that there is little or no tonic suppression or function by ROS under normal conditions.

To determine whether superoxide plays a mechanistic roll in blood pressure-associated vascular endothelial dysfunction, FMD_{BA} was measured before (saline infusion) and after intravenous administration of ascorbic acid (American Regent Laboratories Inc., Shirley, NY). A priming bolus of 0.06 $g \cdot kg^{-1}$ fat-free mass dissolved in 100 ml of saline was infused in an antecubital vein at 5 $ml \cdot min^{-1}$ for 20 minutes. This was followed by a maintenance drip infusion

of 0.02 $g \cdot kg^{-1}$ fat-free mass dissolved in 30 ml of saline administered over 60 minutes at 0.5 $ml \cdot min^{-1}$; FMD_{BA} was measured during the maintenance drip infusion.

Endothelial cell protein expression via endovascular biopsy. Endothelial cells were collected from an antecubital vein with sterile J-wires briefly advanced (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter, and cells were recovered by washing and centrifugation. Cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine coated slides (Sigma Chemical, St Louis, Mo). Cells were frozen at -70°C until analysis; thus, endothelial cells were collected at the same time as functional (i.e., blood pressure, FMD_{BA}) analyses were performed for each subject. Subjects were not recalled for collection of endothelial cells for the present analysis.

For immunofluorescence staining, cells were rehydrated with PBS and rendered permeable with 0.1% Triton X-100 and nonspecific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, PA, USA). Cells were incubated with monoclonal antibodies for nitrotyrosine (Abcam, Cambridge, UK), NADPH oxidase subunit $p47^{phox}$ (Abcam, Cambridge, UK), MnSOD (Stressgen Biotechnologies, San Diego, CA), and NFκB p65 (Novus, Littleton, CO; Santa Cruz, Dallas, TX), as well as with an AlexaFluor fluorescent secondary antibody (Invitrogen, Carlsbad, CA). Cells were incubated with von Willebrand factor to identify endothelial cells, and with DAPI to confirm nuclear integrity. Cells were stored at 4°C overnight.

Slides were viewed using a fluorescence microscope (Eclipse 600; Nikon, Melville, NY). Fluorescence intensity of the primary antibody-dependent AlexaFluor staining (i.e., average pixel intensity) was analyzed using Metamorph Software (Universal Imaging, Downingtown, PA). Eight slides and two control cultured human umbilical vein endothelial cell (HUVEC; passage 6-9 processed identically to the sample cells) slides were selected for each staining batch. Values are reported as a ratio of sample endothelial cells to HUVEC average pixel

fluorescence intensity to reduce batch-to-batch variability. Reporting ratios of vascular endothelial cell protein expression to HUVECs is standard procedure in our laboratory[12,66–68] and others[69–72].