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Habitually exercising older men do not demonstrate age-associated vascular endothelial oxidative stress

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Summary

We tested the hypothesis that older men who perform habitual aerobic exercise do not demonstrate age-associated vascular endothelial oxidative stress compared with their sedentary peers. Older exercising men (n = 13, 62 ± 2 years) had higher ($P < 0.05$) physical activity (79 ± 7 vs. 30 ± 6 MET hours per week) and maximal exercise oxygen consumption (42 ± 1 vs. 29 ± 1 mL kg^{-1} per minute) vs. sedentary men (n = 28, 63 ± 1 years). Brachial artery flow-mediated dilation (FMD), a measure of vascular endothelial function, was greater ($P < 0.05$) in the exercising vs. sedentary older men (6.3 ± 0.5 vs. 4.9 ± 0.4%Δ) and not different than young controls (n = 20, 25 ± 1 years, 7.1 ± 0.5%Δ). In vascular endothelial cells sampled from the brachial artery, nitrotyrosine, a marker of oxidative stress, was 51% lower in the exercising vs. sedentary older men (0.38 ± 0.06 vs. 0.77 ± 0.10 AU). This was associated with lower endothelial expression of the oxidant enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (p47^{phox} subunit, 0.33 ± 0.05 vs. 0.61 ± 0.09 AU) and the redox-sensitive transcription factor nuclear factor kappa B (NFκB) (p65 subunit, 0.36 ± 0.05 vs. 0.72 ± 0.09 AU). Expression of the antioxidant enzyme manganese superoxide dismutase (SOD) (0.57 ± 0.13 vs. 0.30 ± 0.04 AU) and activity of endothelium-bound extracellular SOD were greater (6.4 ± 0.5 vs. 5.0 ± 0.6 U mL^{-1} per minute) in the exercising men (both $P < 0.05$), but differences no longer were significant after correcting for adiposity and circulating metabolic factors. Overall, values for the young controls differed with those for the sedentary, but not the exercising older men. Older men who exercise regularly do not demonstrate vascular endothelial oxidative stress, and this may be a key molecular mechanism underlying their reduced risk of cardiovascular diseases.

Key words: aging; endothelial function; nicotinamide adenine dinucleotide phosphate oxidase; nitrotyrosine; nuclear factor kappa B; superoxide dismutase.

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Introduction

Increasing age is the major risk factor for cardiovascular diseases (CVD), in part because of the development of vascular endothelial dysfunction (Lakatta & Levy, 2003). Consistent with this, sedentary older men demonstrate impaired endothelium-dependent dilation (Celermajer *et al.*, 1994; Donato *et al.*, 2007; Pierce *et al.*, 2011), a key feature of endothelial dysfunction and an independent predictor of future clinical CVD events (Yeboah *et al.*, 2007, 2009).

Pharmacological findings in humans suggest that vascular endothelial dysfunction with aging is mediated by oxidative stress (Taddei *et al.*, 2001). Using a novel translational approach, we recently provided direct evidence for endothelial oxidative stress with human aging. Vascular endothelial cells obtained from older men without CVD had higher staining for nitrotyrosine, a cellular footprint of oxidative stress, compared with cells from young men (Donato *et al.*, 2007). The cells from older men also had greater expression of the p47^{phox} subunit of the oxidant-producing enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, as well as the p65 subunit of the redox-sensitive pro-oxidant/pro-inflammatory transcription factor, nuclear factor kappa B (NFκB).

In contrast to their sedentary peers, older men who perform regular aerobic exercise have largely preserved vascular endothelial function (Rinder *et al.*, 2000; Eskurza *et al.*, 2004, 2005; Franzoni *et al.*, 2005; Pierce *et al.*, 2011) and reduced CVD risk (Blair *et al.*, 1996; Sui *et al.*, 2007). Although pharmacological data suggest reduced oxidative stress is the likely mechanism of action (Taddei *et al.*, 2000; Eskurza *et al.*, 2004), there is no direct cellular evidence that older men who exercise have lower vascular endothelial oxidative stress compared with older sedentary men.

In the present study, we tested this hypothesis and determined whether the absence of endothelial oxidative stress in older exercising men is associated with lower endothelial expression of NADPH oxidase and NFκB. Because the preserved endothelial function in old mice that exercise is associated with enhanced vascular manganese and extracellular superoxide dismutase (SOD) expression and/or activities (Durrant *et al.*, 2009), we also determined whether these antioxidant enzymes were elevated in older exercising men. To address these issues, we assessed brachial artery FMD, a clinically important measure of endothelium-dependent dilation (Yeboah *et al.*, 2009), vascular endothelial cells obtained from brachial artery sampling and endothelium-bound extracellular SOD activity in older sedentary and exercising men. A group of young adult controls were also assessed to provide normal reference values.

Results

Subject characteristics

Physical activity and resting heart rate were similar in the older sedentary men and young controls, but maximal oxygen consumption, a measure of maximal aerobic exercise capacity, was lower ($P < 0.05$) in the older sedentary group (Table 1). The older exercising men had higher physical activity levels and lower resting heart rate compared with the other two

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Table 1 Subject characteristics

	Young sedentary (n = 20)	Older sedentary (n = 28)	Older exercising (n = 13)
Age (years)	25 ± 1	63 ± 1*	62 ± 2*
VO_2 max (mL kg^{-1} per minute)	41 ± 2	29 ± 1*	42 ± 1†
Leisure physical activity (MET hours per week)	37 ± 7	30 ± 6	79 ± 7*†
Heart rate (beats min^{-1})	64 ± 2	61 ± 2	54 ± 2*†
Body weight (kg)	79 ± 2	84 ± 3	73 ± 1*†
Body mass index (kg m^{-2})	26 ± 1	27 ± 0.5	23 ± 0.4*†
Waist/hip ratio	0.85 ± 0.01	0.94 ± 0.01*	0.88 ± 0.01†
Systolic blood pressure (mmHg)	118 ± 3	124 ± 2	120 ± 2
Diastolic blood pressure (mmHg)	69 ± 2	78 ± 1*	74 ± 2*
Total cholesterol (mg dL^{-1})	163 ± 6	196 ± 3*	195 ± 5*
LDL cholesterol (mg d^{-1})	96 ± 5	124 ± 3*	117 ± 4*
HDL cholesterol (mg dL^{-1})	42 ± 2	49 ± 2*	60 ± 3*†
Triglycerides (mg dL^{-1})	122 ± 11	114 ± 6	89 ± 8*†
Glucose (mg dL^{-1})	87 ± 1	94 ± 1*	89 ± 2†
Insulin (μU mL^{-1})	7.7 ± 1	7.2 ± 0.7	5.5 ± 0.9
Oxidized LDL (U L^{-1})	47 ± 4	60 ± 2*	51 ± 5
C-reactive protein (mg L^{-1})	1.1 ± 0.3	1.2 ± 0.2	0.7 ± 0.2

Data are mean ± standard error.
VO_2 max, rate of oxygen consumption at maximal exercise; MET, metabolic equivalent; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

groups. Maximal oxygen consumption was greater ($P < 0.05$) in the older exercising men compared with the older sedentary men and similar to values in the young controls. These results establish higher physical activity levels and associated physiological differences in the exercising compared with sedentary older men.

All of the groups had clinically normal values for body weight, body mass index, waist/hip ratio, blood pressure, and circulating metabolic factors (Table 1). As expected, modest differences were observed in selective characteristics across age in the sedentary men and between the sedentary and exercising older groups.

Plasma-oxidized low-density lipoprotein, a circulating marker of oxidative stress, was greater ($P < 0.05$) in the older sedentary men compared with the young controls, but did not differ between the older exercising men and young controls (Table 1). Plasma C-reactive protein did not differ among the groups.

Brachial artery endothelial function

Brachial artery flow-mediated dilation (FMD) was lower ($P < 0.05$) in the sedentary older men compared with the young controls, whereas older exercising men had greater ($P < 0.05$) brachial FMD than their sedentary peers (Table 2). There were no group differences in baseline diameter or shear rate (older groups only). Endothelium-independent dilation of the brachial artery in response to sublingual nitroglycerin was similar among the groups, indicating that the age- and exercise-associated differences in brachial FMD were attributable to differences in the vascular endothelium *per se*.

Brachial artery endothelial cell oxidative stress and associated molecular influences

Staining for nitrotyrosine was greater ($P < 0.05$) in endothelial cells obtained from the brachial artery of older sedentary men compared with the young controls (0.77 ± 0.10 vs. 0.45 ± 0.06 intensity/HUVEC). Endothelial cell nitrotyrosine was ~50% lower in the exercising compared with the sedentary older men (0.38 ± 0.06) and was similar to that of the young men (Fig. 1).

Compared with the young controls, the greater endothelial cell nitrotyrosine in the older sedentary men was associated with greater ($P < 0.05$) expression of NADPH oxidase $p47^{phox}$ (0.61 ± 0.09 vs. 0.34 ± 0.02 intensity/HUVEC) and NFκB p65 (0.72 ± 0.09 vs. 0.50 ± 0.04 intensity/HUVEC), and lower ($P < 0.05$) expression of man-

Table 2 Brachial artery function

	Young sedentary (n = 20)	Older sedentary (n = 28)	Older exercising (n = 13)
Baseline diameter (mm)	4.0 ± 0.09	4.1 ± 0.1	3.8 ± 0.09
Baseline shear rate (s^{-1})	–	14 ± 2	18 ± 7
Flow-mediated dilation (FMD) (%Δ)	7.1 ± 0.5	4.9 ± 0.4*	6.3 ± 0.5†
Flow-mediated dilation (mmΔ)	0.28 ± 0.02	0.20 ± 0.01*	0.24 ± 0.02†
Peak shear rate during FMD (s^{-1})	–	104 ± 5	109 ± 9
Dilation to sublingual GTN (%Δ)	25 ± 2 (n = 16)	25 ± 1 (n = 18)	27 ± 2 (n = 8)
Dilation to sublingual GTN (mmΔ)	0.97 ± 0.07 (n = 16)	0.99 ± 0.05 (n = 18)	1.02 ± 0.06 (n = 8)

Data are mean ± standard error.
GTN, glyceryl trinitrate.
*$P < 0.05$ vs. young sedentary; †$P < 0.05$ vs. older sedentary.

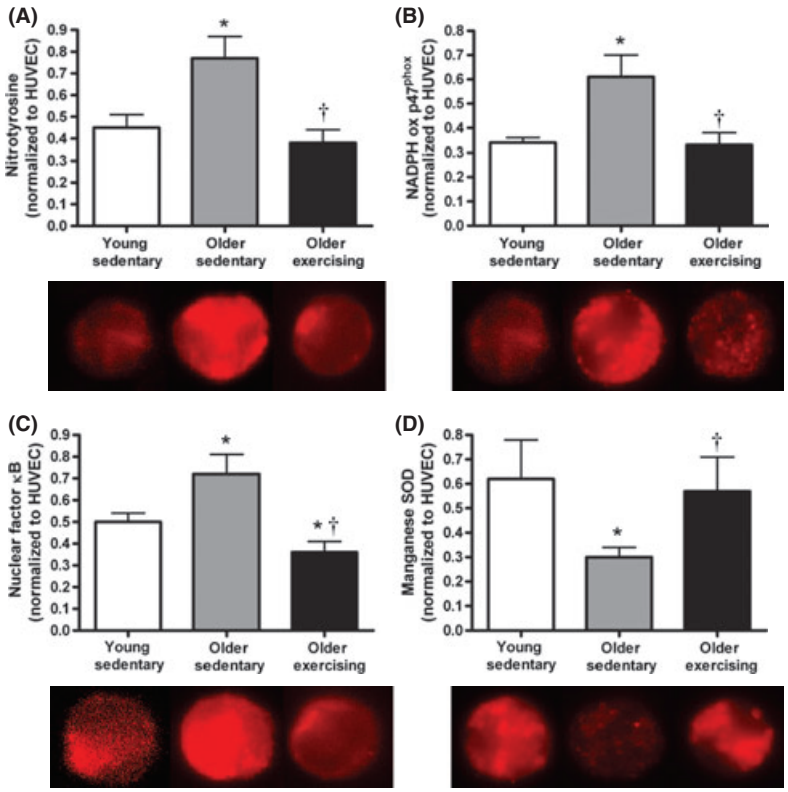

Fig. 1 Arterial endothelial cell nitrotyrosine (top left panel), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p47^{phox} (top right panel), nuclear factor kappa B (bottom left panel), and manganese superoxide dismutase (SOD, bottom right panel) in young sedentary, older sedentary, and older exercising men. Data are normalized to human vascular endothelial cell (HUVEC) protein expression via immunoflourescence. *$P < 0.05$ vs. young sedentary; †$P < 0.05$ vs. older sedentary.

ganese SOD (0.30 ± 0.04 vs. 0.62 ± 0.16 intensity/HUVEC; Fig. 1). In contrast, expression of these oxidant stress-modulating factors was similar in the older exercising men and young controls. NADPH oxidase p47^{phox} (0.33 ± 0.05) and NFκB p65 (0.36 ± 0.05) were 85% and 100% lower ($P < 0.05$) in endothelial cells from the exercising compared with the sedentary older men, respectively, whereas MnSOD (0.57 ± 0.14) was 90% higher ($P < 0.05$; Fig. 1).

Group differences in endothelial cell nitrotyrosine, NADPH oxidase p47^{phox}, and NFκB p65 all remained significantly different (all $P < 0.05$) after covarying for body fatness (i.e., body mass index, total body fat%, waist circumference, and waist/hip ratio) and circulating metabolic factors [low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and total/HDL cholesterol ratio] (Table 1). However, differences in manganese SOD expression between the sedentary and exercising older men no longer were significant after correcting for differences in body fatness ($P = 0.89$) and circulating metabolic factors ($P = 0.11$).

Endothelium-bound extracellular SOD activity

Endothelium-bound extracellular SOD activity was lower ($P < 0.05$) in the sedentary older men compared with the young controls (5.0 ± 0.6 vs. 6.9 ± 0.4 U mL^{-1} per minute), but was preserved in the exercising older men (6.4 ± 0.5; Fig. 2). The differences between the groups of older men no longer were significant after correction for body fatness ($P = 0.07$) and circulating metabolic factors ($P = 0.07$), although a strong trend remained.

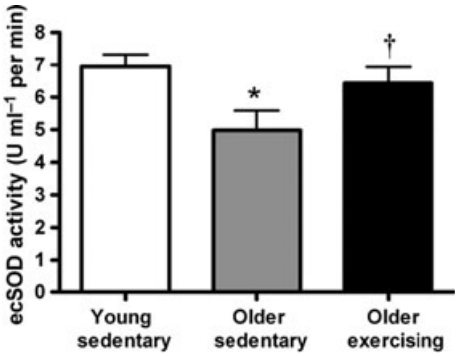

Fig. 2 Endothelium-bound extracellular superoxide dismutase (ecSOD) activity in young sedentary, older sedentary, and older exercising men. *$P < 0.05$ vs. young sedentary; †$P < 0.05$ vs. older sedentary.

Discussion

We used a unique translational approach involving sampling and analysis of endothelial cells obtained from arteries of humans to investigate the molecular events underlying the preserved vascular endothelial function associated with physically active aging. The results provide the first direct evidence that, in contrast to their sedentary peers, older men who perform regular aerobic exercise do not demonstrate age-related vascular endothelial oxidative stress. The latter is associated with an apparent prevention of the increases in the oxidant enzyme NADPH oxidase and the redox-sensitive transcription factor NFκB and decreases in the antioxidant

enzyme manganese SOD in endothelial cells, as well as maintenance of endothelium-bound extracellular SOD activity. Indeed, the exercising men had an overall vascular endothelial cell oxidative phenotype similar to young men. As such, our findings may provide novel insight into the molecular mechanisms underlying the preserved vascular endothelial function and reduced risk of CVD in older men who exercise.

Oxidant stress

Recently, we showed that nitrotyrosine staining is increased in vascular endothelial cells of older compared with young sedentary healthy men (Donato *et al.*, 2007). Here, we extend those findings by showing that vascular endothelial cells from older men who regularly perform aerobic exercise do not demonstrate an increase in this cellular signature of oxidative stress. This is consistent with a recent report from our laboratory showing increased aortic nitrotyrosine abundance and vascular endothelial dysfunction in old compared with young cage-restricted mice, but not in old animals given access to voluntary running wheels for 10–14 weeks (Durrant *et al.*, 2009). The present findings are also in agreement with pharmacological studies, suggesting that the preserved vascular endothelial function in older men who exercise is mediated by reduction in oxidative stress (Taddei *et al.*, 2000; Eskurza *et al.*, 2004). Our results here provide the first molecular evidence in humans supporting this notion. Although not specific to endothelial function, age- and exercise-associated differences in plasma low-density lipoprotein, a circulating marker of oxidative stress, were consistent with the differences in endothelial cell nitrotyrosine.

NADPH oxidase and NFκB

The lack of increases in NADPH oxidase and NFκB in endothelial cells of the exercising older men in the present study may be among the mechanisms contributing to the absence of oxidative stress. NADPH oxidase is a major source of superoxide production in the vasculature (Lassegue & Griendling, 2010), whereas NFκB responds to increased superoxide by stimulating pro-oxidant and pro-inflammatory gene expression that reinforces a state of oxidant stress (Helenius *et al.*, 1996; Ungvari *et al.*, 2007). Vascular expression/activation of NADPH oxidase and NFκB are increased with age in humans (Donato *et al.*, 2007, 2008) and rodents (Durrant *et al.*, 2009; Lesniewski *et al.*, 2009). The present findings advance this previous work by showing that these pathways are not upregulated in older men who exercise regularly. This is consistent with recent work from our laboratory in old mice given access to running wheels (Lesniewski *et al.*, 2011) as well as data from patients with coronary artery disease who undergo aerobic exercise training (Adams *et al.*, 2005). It also is possible that reductions in superoxide production from other sources in the vascular wall such as mitochondria and uncoupled endothelial nitric oxide synthase may have contributed to the observed decreases in endothelial oxidative stress in the exercising older men.

Manganese and extracellular SOD

Maintenance of endothelial-linked antioxidant systems may also contribute to the resistance to vascular endothelial oxidative stress observed in the older exercising men in the present study. We found that manganese SOD, the mitochondrial isoform of SOD and an important antioxidant enzyme in arteries (Faraci & Didion, 2004; Davidson & Duchen, 2007), was preserved in vascular endothelial cells of the exercising men. Moreover, the activity of endothelium-bound extracellular SOD, a key antioxidant enzyme involved in vascular redox control (Fukai *et al.*, 2002; Faraci & Didion, 2004), also was maintained in older exercising men at levels similar to young controls. These findings are generally consistent with recent observations in mice (Moien-Afshari *et al.*, 2008; Durrant *et al.*, 2009) and emphasize the potential importance of these antioxidant defenses in the preservation of vascular endothelial function with aging in men who exercise.

Limitations

The results of the present study provide only limited insight into the mechanisms underlying the absence of endothelial cell oxidative stress in older men who exercise regularly. Correction for conventional CVD risk factors such as total and abdominal body fatness and circulating metabolic factors did not influence group differences in nitrotyrosine, suggesting that the protection against the development of oxidative stress was not secondary to a lower risk factor burden. Similarly, maintenance of lower expression of NADPH oxidase and NFκB in endothelial cells of the exercising men was not dependent on a more favorable CVD risk factor profile, although correction for body fatness and circulating lipoproteins rendered group differences in antioxidant enzymes (endothelium-bound extracellular SOD activity and endothelial cell manganese SOD) nonsignificant. It is, therefore, possible that regular aerobic exercise induces a primary protective effect against endothelial oxidative stress in older men, perhaps linked to increases in laminar shear associated with systemic hemodynamic changes during exercise (Green, 2009). A secondary effect of exercise via reductions in specific CVD risk factors such as body fatness and circulating lipids may act as another influence lessening endothelial oxidative stress, perhaps in part via modulation of antioxidant enzymes. Moreover, our results pertain only to men without CVD and other clinical disorders. The effect of regular aerobic exercise status on vascular endothelial function may be different in postmenopausal women (Pierce *et al.*, 2011) or in patients with chronic disease (Gokce *et al.*, 2002; Braith *et al.*, 2008). Finally, the relatively small number of arterial endothelial cells obtained from the technique employed limited the number of analyses that could be performed.

Summary and conclusions

In striking contrast to their sedentary peers, older men who exercise regularly do not demonstrate evidence of vascular endothelial oxidative stress. Endothelial cells from these exercising men do not show age-related increases in NADPH oxidase or NFκB, but rather maintenance of mitochondrial and extracellular endothelial SOD. Because endothelial oxidative stress is a major causal factor in vascular pathophysiology and clinical disease (Cai & Harrison, 2000), our results may provide unique insight into the molecular mechanisms underlying the protective effect of aerobic exercise in preserving endothelial function and reducing the risk of developing CVD with aging.

Experimental procedures

Subjects

A total of 61 healthy men were studied: 28 sedentary and 13 exercising middle-aged and older men, and 20 young sedentary controls (Table 1). Subjects were nonobese (body mass index, BMI, < 30 kg m^{-2}), nonsmokers, nondiabetic, and free of other clinical diseases as assessed by medical history, physical examination, blood chemistry, and resting and exercise 12-lead ECG. Subjects were excluded if they were taking any prescription medications, herbal supplements, antioxidants, or aspirin.

Subjects had clinically normal blood pressure, fasting circulating lipids, and glucose. Sedentary men performed no regular aerobic exercise (i.e., ≤ 30 min per day ≤ 2 days per week) for at least the last 2 years. The exercise-trained men performed regular vigorous aerobic exercise (competitive running, triathlons, and/or cycling) ≥ 5 days per week for ≥ 45 min per session for at least the last 5 years. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Measurements

All measurements were taken at the University of Colorado at Boulder Clinical Translational Research Center (CTRC) after a 12-h overnight fast and 24-h abstention from alcohol and physical exercise.

Subject characteristics

Subject characteristics were measured as described previously (Christou *et al.*, 2005). Body mass index was calculated from height and weight to the nearest 0.1 kg, and waist and hip circumferences by anthropometry. Total body fat was determined using dual X-ray absorptiometry (DPX-IQ; GE/Lunar, Madison, WI, USA). All blood assays were performed by the University of Colorado CTRC core laboratory using standard methods (Pierce *et al.*, 2009, 2011).

Brachial artery function

Brachial artery FMD was determined using duplex ultrasonography (Powervision 6000; Toshiba, Inc., New York, NY, USA) with a linear array transducer as described previously by our laboratory (Eskurza *et al.*, 2004, 2005, 2006; Donato *et al.*, 2007; Pierce *et al.*, 2009, 2011). Peak shear rate after forearm occlusion was assessed in a subset of the older sedentary (n = 19) and exercise-trained (n = 6) men. Responses are expressed in millimeters and percentage of maximal change from baseline diameter per recent recommendations (Donald *et al.*, 2008). Endothelium-independent dilation in response to 0.4 mg sublingual nitroglycerin was assessed in subgroups as a control measure of vascular smooth muscle responsiveness to nitric oxide (Table 1).

Endothelial cell analyses

Collection and analyses of arterial endothelial cells from the brachial artery were performed as described previously by our laboratory (Donato *et al.*, 2007, 2008; Silver *et al.*, 2007; Pierce *et al.*, 2009). Following brachial artery catheterization by a CTRC physician using strict aseptic procedures, two sterile J wires (Daig Corp., Minnetonka, MN, USA) were advanced into the brachial artery (~4 cm beyond the tip of the catheter) and retracted through an 18-gauge catheter on a different day than the brachial artery FMD. The two wires plus the catheterization guide wire were then transferred to a dissociation buffer solution, and cells were recovered after a washing and centrifugation protocol. Collected cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine-coated slides (Sigma Chemical, St. Louis, MO, USA) and then frozen at −80°C until analysis.

After blocking nonspecific binding sites with 5% donkey serum (Jackson Immunoresearch, West Grove, PA, USA), cells were incubated with monoclonal antibodies for NFκB p65 (both Novus, Littleton, CO, USA), nitrotyrosine, NADPH oxidase $p47^{phox}$ (Abcam, Cambridge, MA, USA), and manganese SOD (Stressgen, Inc. Farmingdale, NY, USA). Next, cells were incubated with vWF (von Willebrand factor; 1:1000; Dako, Carpinteria, CA, USA) and a specific AlexaFlour488-conjugated secondary antibody (Research Diagnostics, Acton, MA, USA). Slides were then coverslipped with a VECTASHIELD DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) fluorescent mounting medium (Vector Labs, Burlingame, CA, USA) and stored at 4°C overnight. Slides were viewed using a fluorescence microscope (Eclipse 600; Nikon, Melville, NY, USA), and 30 individual endothelial cell images were digitally captured by a Photometrics CoolSNAPfx digital camera (Roper Scientific, Tuscon, AZ, USA). These endothelial cells were documented by cell staining of vWF, and nuclear integrity was confirmed using DAPI staining. Once endothelial cells with intact nuclei were identified, they were analyzed using Metamorph Software (Universal Imaging, Downingtown, PA, USA) to quantify the intensity of primary antibody-dependent AlexaFlour555 staining (i.e., average pixel intensity). The number of cells typically recovered from each guide wire results in approximately 50–100 cells per slide. 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 variability between staining batches. A singe technician was blinded to the identity of the subject during the staining and analysis procedures. Reproducibility is reported elsewhere (Donato *et al.*, 2008).

Extracellular superoxide dismutase (ecSOD) activity

Circulating endothelium-bound extracellular SOD activity was measured in a subset of older sedentary (n = 11) and endurance exercise-trained (n = 13), and young sedentary controls (n = 4) as previously described (Landmesser *et al.*, 2002). Venous blood was collected in chilled EDTA tubes at baseline and at 7, 10, and 15 min after intravenous injection of 5000 U of heparin sulfate, and plasma was frozen immediately at −80°C until analysis. Activity was measured for each sample using a colorimetric assay (Cell Technology, Inc., Mountain View, CA, USA), and area under the activity x time integral was calculated using the trapezoidal method (Matthews *et al.*, 1990).

Data analysis

All data are presented as mean ± standard error. Main effects were determined by one-way ANOVA, with least significant differences *post hoc* analyses used to determine differences between specific groups. ANCOVA was used to determine the effects of differences in body composition and circulating metabolic factors between sedentary and exercising older men on corresponding differences in the primary outcome variables. Statistical significance was set *a priori* at $P < 0.05$. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

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Author contributions

Gary L. Pierce, Douglas R. Seals, and Anthony J. Donato contributed to the conception, experimental design, and interpretation of data for this study. Gary L. Pierce, Iratxe Eskurza, and Annemarie Silver performed brachial artery reactivity studies and collected and processed endothelial cell samples. Gary L. Pierce analyzed all brachial artery data and was blinded to subject group. Thomas J. LaRocca performed the plasma ecSOD activity assays and was blinded to subject group. The manuscript was written by Gary L. Pierce and Douglas R. Seals; however, all authors read, edited, and approved the final version of manuscript.

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