1-15-2016

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Park SY, Ives SJ, Gifford JR, Andtbacka RH, Hyngstrom RH, Reese V, Layec G, Bharath LP, Symons JD, Richardson RS. Impact of age on the vasodilatory function of human skeletal muscle feed arteries. *Am J Physiol Heart Circ Physiol* 310: H217–H225, 2016. First published November 20, 2015; doi:10.1152/ajpheart.00716.2015.

Although advancing age is often associated with attenuated skeletal muscle blood flow and skeletal muscle feed arteries (SMFAs) have been recognized to play a regulatory role in the vasculature, little is known about the impact of age on the vasodilatory capacity of human SMFAs. Therefore, endothelium-dependent and -independent vasodilation were assessed in SMFAs (diameter: 544 ± 63 μm) obtained from 24 (equally represented) young (33 ± 2 yr) and old (71 ± 2 yr) subjects in response to three stimuli: *1*) flow-induced shear stress, *2*) ACh, and *3*) sodium nitropusside (SNP). Both assessments of endothelium-dependent vasodilation, flow (young subjects: 68 ± 1% and old subjects: 32 ± 7%) and ACh (young subjects: 92 ± 3% and old subjects: 73 ± 4%), were significantly blunted ($P < 0.05$) in SMFAs of old compared with young subjects, with no such age-related differences in endothelium-independent vasodilation (SNP). In response to an increase in flow-

induced shear stress, vasodilation kinetics (time constant to reach 63% of the amplitude of the response: 55 ± 1 s in young subjects and 92 ± 7 s in old subjects) and endothelial nitric oxide synthase (eNOS) activation (phospho-eNOS^{s1177}/total eNOS: 1.0 ± 0.1 in young subjects and 0.2 ± 0.1 in old subjects) were also significantly attenuated in old compared with young subjects ($P < 0.05$). Furthermore, the vessel superoxide concentration was greater in old subjects (old subjects: 3.9 ± 1.0 area under curve/mg and young subjects: 1.7 ± 0.1 area under the curve/mg, $P < 0.05$). These findings reveal that the endothelium-dependent vasodilatory capacity, including vasodilation kinetics but not smooth muscle function, of human SMFAs is blunted with age and may be due to free radicals. Given the potential regulatory role of SMFAs in skeletal muscle blood flow, these findings may explain, at least in part, the often observed attenuated perfusion of skeletal muscle with advancing age that may contribute to exercise intolerance in the elderly.

Keyword:

human skeletal muscle feed artery; endothelium-dependent vasodilation; aging

NEW & NOTEWORTHY

This study has identified that a regulatory role of human skeletal muscle feed arteries in skeletal muscle blood flow, and this regulatory function of human skeletal muscle feed arteries is significantly attenuated with advancing age, which is mainly due to blunted endothelium-mediated vascular function.

ATTENUATED CARDIOVASCULAR FUNCTION, as characterized by impaired O_2 delivery to skeletal muscle, is a well-documented phenomenon associated with aging (24, 33, 38) and is likely responsible, at least in part, for the diminished exercise capacity in the elderly. Certainly, with advancing age, due to a decrease in both maximum heart rate and stroke volume, maximum cardiac output is compromised (11, 33, 48). Interestingly, not as a direct consequence, but in addition to this attenuated central cardiovascular response, although not universal, many studies have also documented attenuated leg blood flow and vascular conductance during exercise in the old compared with the young (25, 30, 37, 39, 47). However, the exact location in the vasculature and the mechanisms responsible for this diminished peripheral blood flow response are currently not well understood.

In animals, it has been well documented that skeletal muscle feed arteries (SMFAs), the inlets to the muscle bed upstream of the arterioles, due to location and vasoactive capacity, are primary blood flow regulators during physical activity (41, 49). Furthermore, additional animal work suggests that aging impairs endothelium-dependent dilation in SMFAs (50). Building on these animal studies, Ives et al. (19, 21) recently translated these observations using a pharmacological approach, providing evidence that human SMFAs also have the potential to regulate skeletal muscle perfusion. However, although the likely importance of human SMFAs has been highlighted, few such studies have been performed because these vessels are difficult to obtain, and, therefore, little is currently known about the impact of age on SMFA vasomotor function.

Using in vitro methods to study animal skeletal muscle arterioles, which are

downstream of SMFAs, Muller-Delp et al. (32, 33) documented that local muscle blood flow was reduced in old rats and proposed that the mechanism responsible for this age-related attenuation was impaired endothelium-dependent vasodilation. Additionally, Behnke et al. (2) recently revealed that the rate of skeletal muscle arteriole vasodilation, likely reflective of the kinetics of muscle perfusion within the muscle, is blunted in old compared with young mice. There- fore, in terms of the age-related reduction in O_2 delivery to skeletal muscle, these and other studies have identified a role for diminished nitric oxide (NO) bioavailability and, subsequently, endothelium-mediated vasodilatory capacity in skeletal muscle resistance arteries and arterioles in animal models (9, 16, 33). However, whether endothelial dysfunction associated with advancing age, at the level of the SMFA in humans, contributes to the diminished peripheral blood flow associated with the elderly remains to be determined.

Consequently, using pressure myography, the present study sought to further examine the vasomotor function of human SMFAs with a specific focus on the impact of age. We tested the following hypotheses: first, supporting the concept that SMFAs have regulatory potential, both young and old human SMFAs will exhibit significant vasodilatory capacity when stimulated by flow-induced shear stress, ACh, and sodium nitroprusside (SNP). Second, the endothelium-mediated vasodilatory response, stimulated by flow-induced shear stress and ACh, will be attenuated in old compared with young subjects, whereas the endothelium-independent vasodilatory response (SNP) will not be affected by age. Third, likely due to impaired endothelium-dependent vasodilation, the kinetics of flow-induced vasodilation will be slower in old compared with young subjects. Finally, the reduced endothelium-mediated response with age will

be associated with increased free radicals and/or reduced activation of endothelial NO synthase (eNOS). If these hypotheses are proven to be correct, these findings will add to the understanding of skeletal muscle blood flow regulation with advancing age and have implications for the targeting of interventions aimed at maintaining physical function in the ever-growing elderly population.

METHODS

Subjects and general procedures.

A total of 40 SMFAs from the axillary and inguinal regions were obtained from young (33 ± 2 yr, $n = 20$) and old (71 ± 2 yr, $n = 20$) subjects during melanoma-related surgeries. Twenty-four (equally represented) young and old SMFAs were used in all protocols except the Western blot analyses after only flow exposure, which were performed in the 16 additional SMFAs (8 young SMFAs and 8 old SMFAs). The contribution of NO to both flow and ACh-induced vasodilation in SMFAs was assessed in a subset of 12 vessels (6 young vessels and 6 old vessels) using the NOS inhibitor *N*-monomethyl-L-arginine (L-NMMA). All subjects were free from cancer and chemotherapy, but there were no other specific exclusion criteria for this study, although all medical conditions and medications were noted. All protocols were approved by the Institutional Review Board of the University of Utah and Salt Lake City Veterans Affairs Medical Center and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects before surgery.

Vessel harvest and preparation.

SMFAs (outer diameter: ~500 μm and length: 2–3.0 mm) from the axillary (e.g., serratus anterior or latissimus dorsi muscles) and inguinal (e.g., hip adductors or quadriceps femoris muscles) regions, obtained during sentinel node biopsy for melanoma surgery at the Huntsman Cancer Hospital and the Salt Lake City Veterans Affairs Medical Center, were studied. Patients were anesthetized using a general protocol: propofol, fentanyl, benzodiazepines, and succinylcholine (35). SMFAs were harvested after dissecting out sentinel lymph nodes for clinical analysis and were identified and classified based on being a vascular inlet into a muscle bed, structure, coloration, and pulsatile bleed pattern (20). SMFAs were ligated, excised, and immediately placed in iced normal physiological saline solution (PSS) before transfer to the laboratory within 15 min of being harvested (21).

Vessel function protocols.

SMFAs from 24 (equally represented) young and old subjects were assessed in these protocols. Initially, perivascular adipose and/or connective tissue around SMFAs were removed under a dissecting microscope (SZX10, Olympus, Center Valley, PA) in cold (4°C) PSS containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS buffer with 1 g/100 ml BSA at pH 7.4. The function of SMFAs was measured using pressure myography organ baths (110p, DMT Systems, Aarhus, Denmark). Arteries were cannulated at both ends with micropipette tips, and vessel outer diameters were recorded under an inverted microscope with a video camera (TS100, Nikon Eclipse, Melville, NY), with data streamed in real time to edge detection software (DMT VAS version 2.0) monitored at a

sampling rate of 1 kHz. Fluid leak was detected by pressurizing the vessel to 60 mmHg, the intraluminal pressure used throughout the study (21, 49), closing the cannulas to the fluid reservoirs, and assessing the capacity to maintain vessel diameter. Arteries free from leaks were then warmed to 37°C, allowed to develop spontaneous tone for a 30-min equilibration period (21), and then underwent vasodilation assessments and, ultimately, Western blot analysis.

Vasodilation assessments.

Vasodilation (in %) was assessed in response to three stimuli: first, to assess the endothelium-mediated vasodilatory response to flow-induced shear stress, intraluminal flow was developed. This was achieved by altering the heights of the independent fluid reservoirs, contiguous with the SMFAs, in equal and opposite directions so that a pressure difference was developed across the vessel without altering the mean intraluminal pressure of 60 mmHg (2). Pilot work revealed a robust increase in vasodilation in response to the flow induced by a pressure difference of 30 mmHg compared with 15 mmHg and more subtle increases in vasodilation from 30 to 44 mmHg and from 44 to 60 mmHg. Hence, a single pressure difference of 30 mmHg, which yielded an approximate flow rate of 30 µl/min, was adopted for all subsequent flow experiments. With this flow rate and typical SMFA internal diameter, an expected shear rate of ~500 s^{-1} was calculated using the following equation: 8 X mean velocity/vessel diameter (34). Additionally, to determine the contribution of NO to the flow-induced percentage of vasodilation, intraluminal flow was developed in a subset of 12 vessels (6 young vessels and 6 old vessels) in the presence and absence of the

NOS inhibitor L-NMMA (10^{-3} M, 30 min), as previously described (46). Second, to assess endothelium-dependent vasodilation pharmacologically, an ACh (10^{-7}–10^{-3} M) dose-response curve was performed after preconstriction with phenylephrine (PE; 10^{-6}–10^{-4} M) to ~70% of the maximum PE response. Again, to determine the contribution of NO to the ACh-induced percentage of vasodilation, an Ach dose-response curve was performed in a subset of 12 vessels (6 young vessels and 6 old vessels) in the presence and absence of L-NMMA (10^{-3} M, 30 min), as previously described (46). Third, to assess endothelium-independent vasodilation, a SNP (10^{-9}–10^{-4} M) dose- response curve was performed after preconstriction with PE (10^{-6}– 10^{-4} M) to ~70% of the maximum PE response. Vasodilation sensitivity was defined as the concentration of ACh or SNP that elicited 50% of the maximal response (EC_{50}), which was calculated by a sigmoidal parameter, as described previously (20). Ca^{2+}-free normal PSS was used to measure the maximum passive relaxation of SMFAs, as previously described (13).

Western blot analysis.

Baseline protein expression of phosphorylated (p-)eNOS at Ser^{1177} (catalog no. 9570, Cell Signaling, Boston, MA), a well-described eNOS activation site (10), and total eNOS (catalog no. 610296, BD Transduction, San Jose, CA) analysis was performed (52) using a subset (8 young arteries and 8 old arteries) of unused pieces of the same arteries that were assessed for vessel function. An additional 16 arteries (8 young arteries and 8 old arteries) were snap frozen in liquid nitrogen immediately after being exposed to only 6 min of flow at ~30 ± 1 μl/min to measure the impact of flow on eNOS

and p-eNOS Ser^{1177} protein expression. GAPDH (ab9485, Abcam, Cambridge, MA) was used as a loading control, and, therefore, the protein data from the Western blot analyses were normalized using GAPDH. Western blots were performed in duplicate, and the data were averaged.

Free radical measurement.

A subset (8 young arteries and 8 old arteries) of unused pieces of the same arteries that were assessed for vessel function were used in this protocol. Electron paramagnetic resonance (EPR) spectroscopy was performed on frozen tissue to directly assess free radical concentration using the superoxide-specific spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroli- dine (CMH; Enzo Life Sciences, Farmingdale, NY), as previously described (5). An EPR spectrometer (EMX X micro, Bruker, Manning Park Billerica, MA) was used in combination with commercially available software (version 1.1b.51, Xenon, Bruker) to calculate the area under the curve (AUC) of the EPR spectroscopy signal by double integration.

Calculations.

Percent vasodilation was used for data expression to account for baseline differences in vessel diameter and calculated using the following equation: (D_T - D_p/D_i - D_p) X 100, where D_T is the recorded diameter at a given time point, D_p is the diameter recorded after the addition of the vasoactive agent (i.e., preconstriction diameter), and D_i is the diameter recorded immediately before the addition of the vasoactive agent (initial diameter) (2).

Vasodilation kinetics were determined by fitting the time-dependent vessel diameter changes during the shear stimulus period to a single exponential curve described by the following equation: diam- eter$_t$ = diameter$_{base}$ + diameter$_{end}$[1 – $e^{-(t - TD/T)}$], where diameter$_t$ is the change in diameter at *time t*, diameter$_{base}$ is the baseline diameter, diameter$_{end}$ refers to the maximal vessel diameter in response to the shear stimulus, TD is the time delay before the onset of diameter change, and T is the time constant to reach 63% of the amplitude of the response (2).

Statistical analyses.

Statistical analyses were performed using SPSS statistical software (SPSS version 17, SPSS, Chicago, IL). Group differences and vessel characteristics were compared using two-way ANOVA. One-way repeated-measures ANOVA was used to determine significant changes in vessel diameter for dose responses to ACh and SNP. A Student's *t*- test was performed to identify the alteration in vessel diameter if the ANOVA was significant. For all analyses, *P* values of <0.05 were considered significantly different. All data are expressed as means ± SE.

RESULTS

Subject characteristics.

A total of 40 subjects participated in this study, with 24 SMFAs harvested and assessed for vasodilatory function and basal eNOS and p-eNOS Ser^{1177} protein expression and superoxide levels assessed in 2 subsets of 16 of these 24 vessels; Western blot analyses, after only flow expo- sure, were performed in the 16 additional

SMFAs. In all assessments, the young and old groups were equally represented. The characteristics of the predominantly Caucasian subjects, obtained from preoperative examination medical re- cords, are shown in Table 1. Note that users of cardiovascular (β-blocker, angiotensin-converting enzyme inhibitor, diuretic, Ca^{2+} channel blocker, etc.), diabetic (insulin, metformin, etc.), and cancer-related medications were excluded from the study. In addition, it should be noted that all blood chemistry and complete blood count results (Table 1) were within the normal range, suggesting that the subjects who participated in this study were relatively healthy.

Vessel characteristics.

SMFAs were harvested from either the inguinal (n = 27) and axial (n = 13) regions, and, in line with our previous observations (20, 21), there were no statistical differences in vasoconstriction or vasodilation responses in terms of both anatomic origin and sex. The basal, unpressurized, outer diameter of SMFAs was not statistically different between young and old SMFAs (young SMFAs: 510 ± 12 μm and old SMFAs: 514 ± 15 μm). Additionally, the maximal outer diameter of SMFAs, achieved by Ca^{2+}-free normal PSS incubation, in young and old SMFAs was not statistically different (young SMFAs: 758 ± 19 μm and old SMFAs: 752 ± 14 μm).

Vasodilatory response to intraluminal flow.

The PE-induced preconstriction of SMFAs before the flow stimulus was similar between groups (old group: 68 ± 5% and young group: 69 ± 4%, $P > 0.05$). The greatest vasodilation in response to the intraluminal flow of 30 ± 1 μl/min was significantly

Table 1. Subject characteristics

	Young Group	Old Group
n	20	20
Age, yr	33±2	71±2*
Sex		
Men, men/total n	17/20	18/20
Women, women/total n	3/20	2/20
Height, cm	174±2	169±9
Body mass, kg	79±10	72±8
Body mass index, kg/m^2	26±4	25±5
Systolic blood pressure, mmHg	118±4	120±5
Diastolic blood pressure, mmHg	75±2	81±2
Glucose, mg/dl	108.1±8.4	109.3±6.8
Blood urea nitrogen, mg/dl	17.4±2.0	16.9±3.0
Creatinine, mg/dl	0.8±0.2	0.9±0.1
Albumin, g/dl	4.2±0.4	4.3±0.5
Lactate dehydrogenase, U/l	501.4±34.3	504.3±36.1
Hemoglobin, g/dl	14.4±0.9	13.5±0.8
White blood cells, K/μl	5.2±0.8	8.4±0.9
Red blood cells, K/μl	4.5±0.3	4.2±0.2
Platelets, K/μl	246.2±20.4	254±30.5
Hematocrit, %	42.8±1.9	40.3±1.3
Lymphocytes, %	30.5±4.0	32.4±2.0
Monocytes, %	8.0±1.6	7.8±1.3
Medications, users/*n*		
Diuretics	0/12	0/12
Angiotensin-converting enzyme inhibitors	0/12	0/12
Diabetic drugs	0/12	0/12
Statins	0/12	2/12

Data are expressed as means ± SE or numbers of subjects (of the total number; *n*). *Significantly different from young subjects, $P < 0.05$.

reduced in the old group compared with the young group (old group: 32 ± 7% and young group: 68 ± 1%, $P < 0.05$; Figs. 1 and 2). T, an indicator of vasodilation kinetics, for the flow-induced vasodilation was significantly slower in the old group com- pared with the young group (old group: 92 ± 7 s and young group: 55 ± 13 s, $P < 0.05$). Furthermore, the time delay before a statistically significant flow-induced change in diameter occurred tended to be slower in the old group compared with the young group

(old group: 8 ± 2 s and young group: 3 ± 1 s) but did not achieve statistical significance (P = 0.07; Fig. 2). Additionally, the comparison between flow-induced vasodilation in the presence and absence of L-NMMA revealed a significant reduction in peak vasodilation, and this L-NMMA- induced change was significantly greater in the young group compared with the old group (old group: change of 27 ± 7% and young group: change of 43 ± 9%, P < 0.05; Fig. 3).

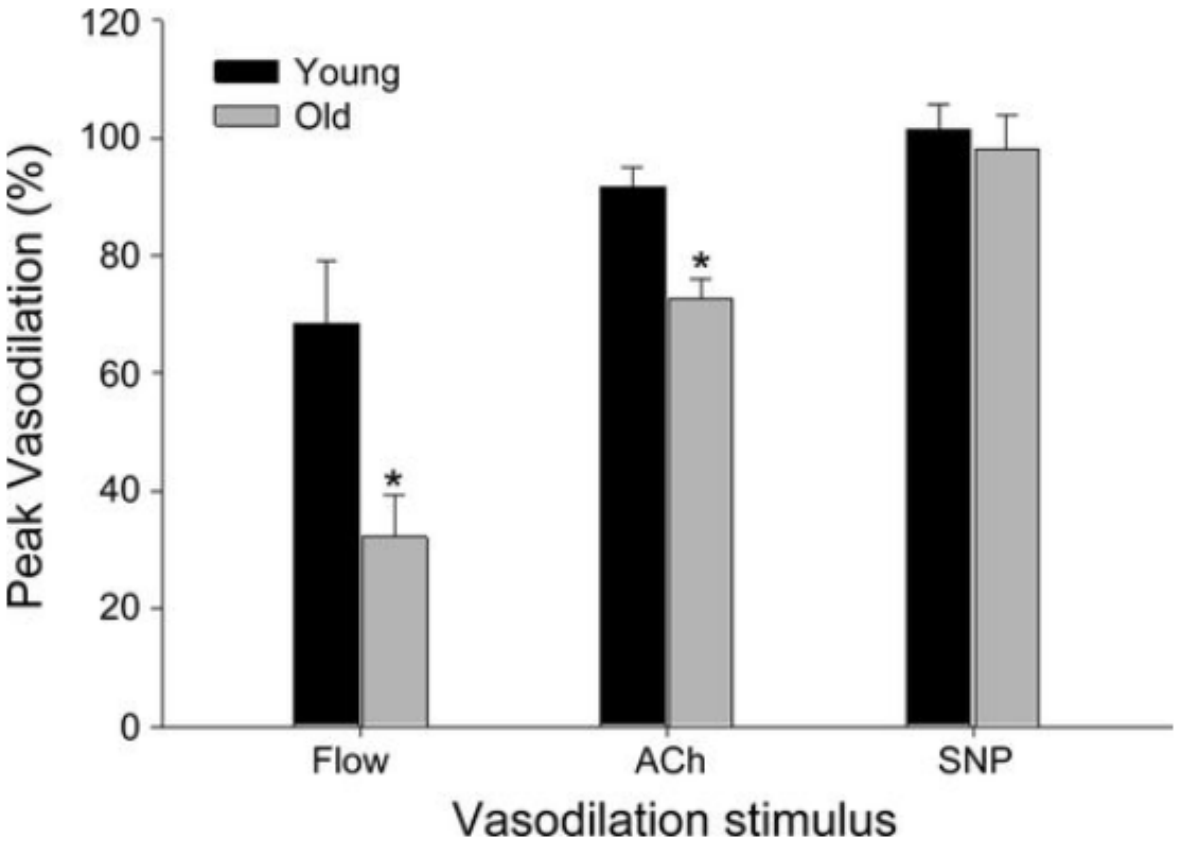

Fig. 1. Skeletal muscle feed artery (SMFA) peak percentage of vasodilation induced by flow, ACh, and sodium nitropruside (SNP) in young and old subjects. Data are expressed as means ± SE; n = 12 young and 12 old subjects. *Significantly different from young subjects, P < 0.05.

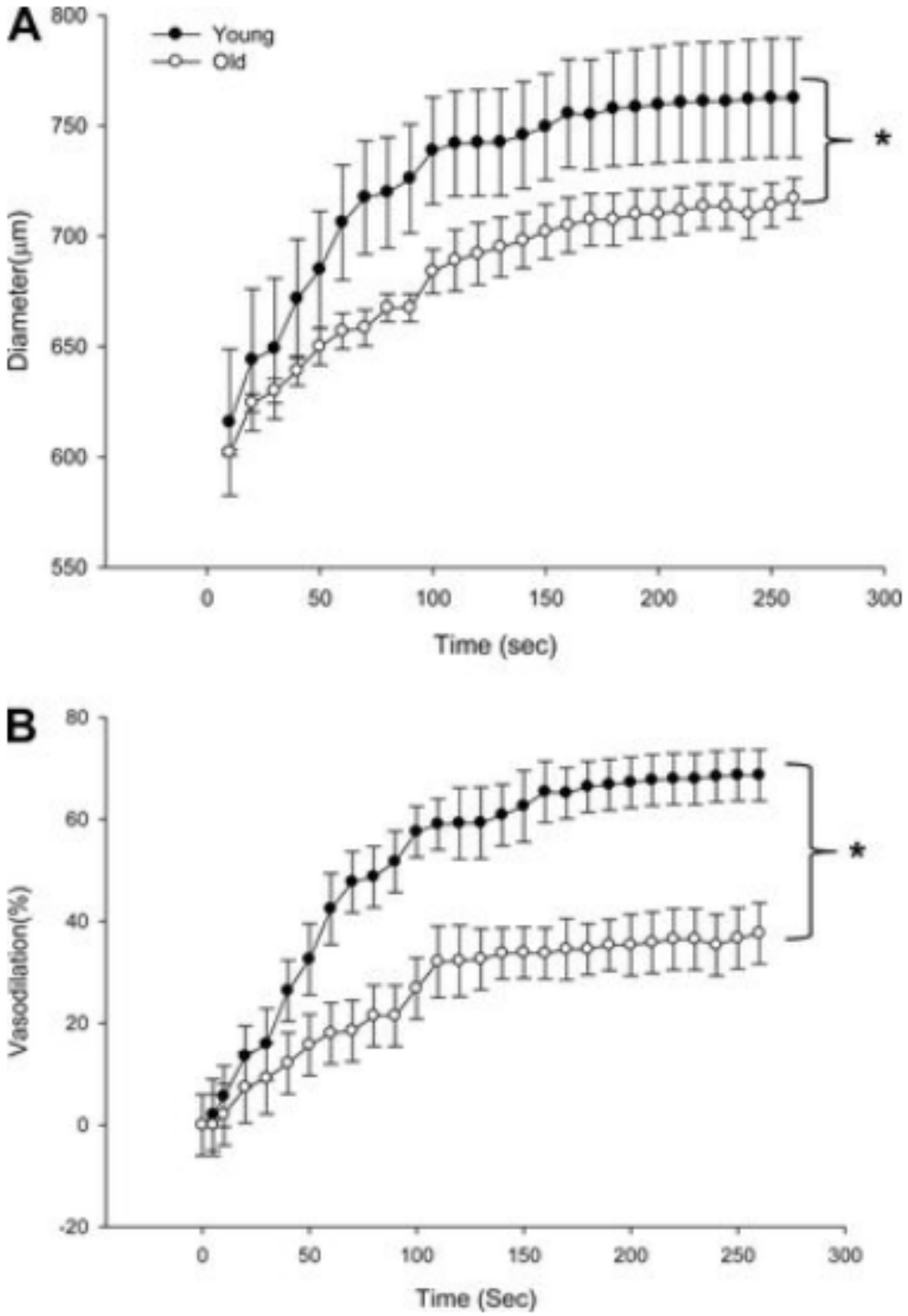

Fig. 2. SMFA vasodilatation kinetics illustrated as absolute changes in diameter (*A*) and percentages of vasodilation (*B*) in young and old subjects. Data are expressed as means ± SE; *n* = 12 young and 12 old subjects. *Significant difference between young and old subjects, $P < 0.05$.

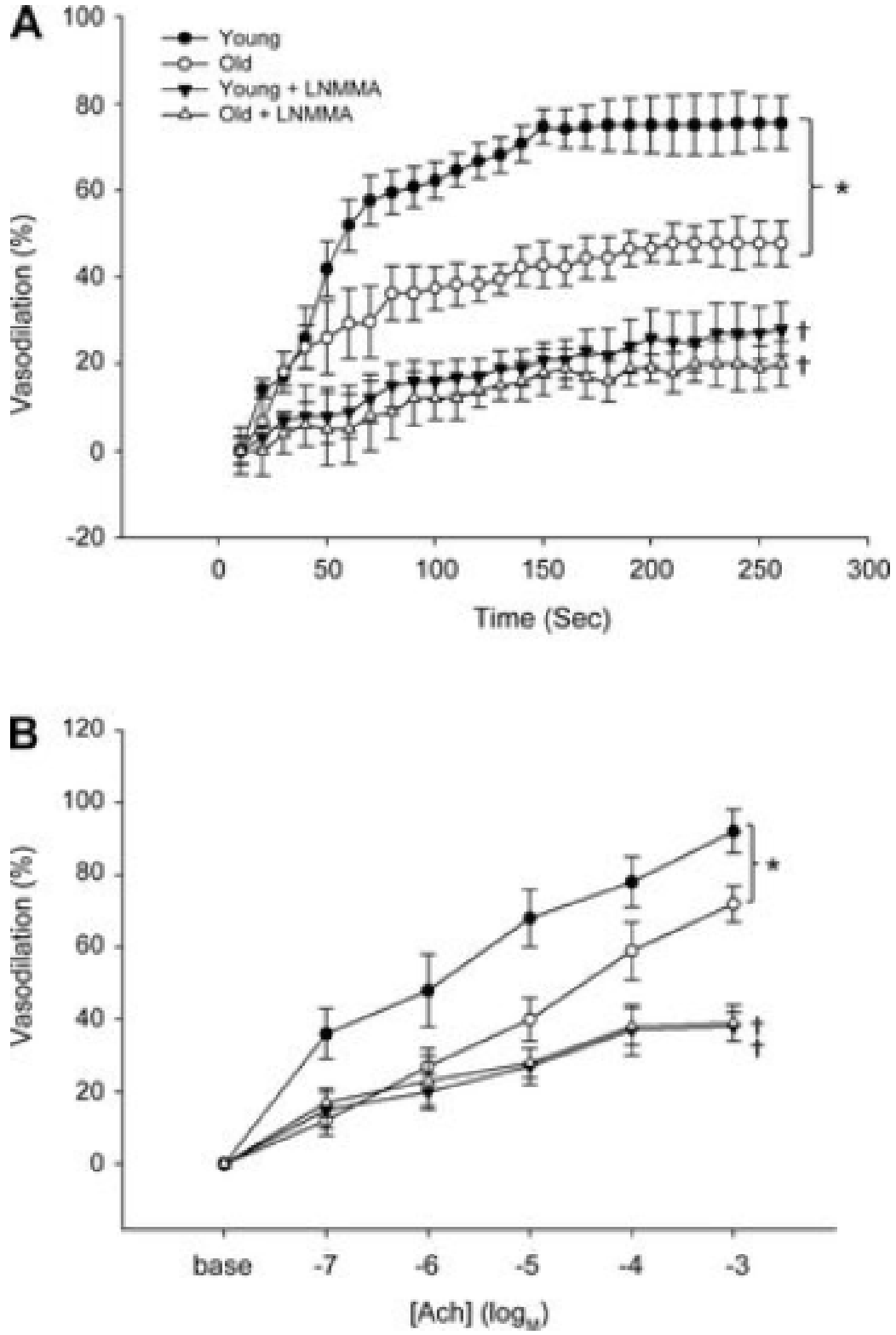

Fig. 3. SMFA vasodilation kinetics in response to flow (*A*) and increasing doses of ACh (*B*) in the absence or presence of the nitric oxide synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NMMA) in young and old subjects. *n* = 6 young and 6 old subjects. *Significant difference between young and old subjects, $P < 0.05$; †Significant difference between flow alone and with L-NMMA, $P < 0.05$.

Vasodilatory responses to ACh and SNP.

PE-induced preconstriction of SMFAs before the both the ACh and SNP vasodilatory stimuli were similar between groups (old group: 68 ± 5% and young group: 69 ± 5%, $P > 0.05$). SMFAs from the old group exhibited significantly attenuated vasodilation in response to the highest dose of ACh (10^{-3} M) in the ACh dose-response curve than the young group (old group: 73 ± 4% and young group: 92 ± 3%, $P < 0.05$; Figs. 1 and 4*A*). In contrast, the greatest vasodilatory response to SNP (10^{-4} M) in the SNP dose-response curve was similar between old and young groups (old group: 112 ± 11% and young group: 102 ± 4%, $P > 0.05$; Figs. 1 and 4*B*). Additionally, the comparison between ACh-induced vasodilation in the presence and absence of L-NMMA revealed a significant reduction in peak vasodilation, and this L-NMMA-induced change was significantly greater in the young group compared with the old group (old group: change of 29 ± 6% and young group: change of 52 ± 4%, $P < 0.05$; Fig. 3). The sensitivity to ACh, as assessed by log EC_{50}, was significantly attenuated in the old group compared with the young group (old group: -4.1 ± 0.4 and young group: -5.5 ± 0.3, $P < 0.05$), whereas the sensitivity to SNP was not different between the old and young groups (old group: -5.75 ± 0.5 and young group: -5.77 ± 0.5, $P > 0.05$; Fig. 4).

Protein expression.

The baseline ratio of p-eNOS Ser^{1177} to total eNOS was significantly greater in the old group compared with the young group (2.8 ± 0.08- and 1.0 ± 0.04-fold change relative to the young group for the old and young groups, respectively, $P < 0.05$; Fig. 5*A*). However, p-eNOS Ser^{1177}, an activation site of eNOS on the Ser^{1177} residue, to total eNOS protein expression in response to a 6-min flow stimulus was significantly

lower in the old group compared with the young group (0.2 ± 0.08- and 1.0 ± 0.04-fold change relative to the young group for the old and young groups, respectively, $P < 0.05$; Fig. 5*B*).

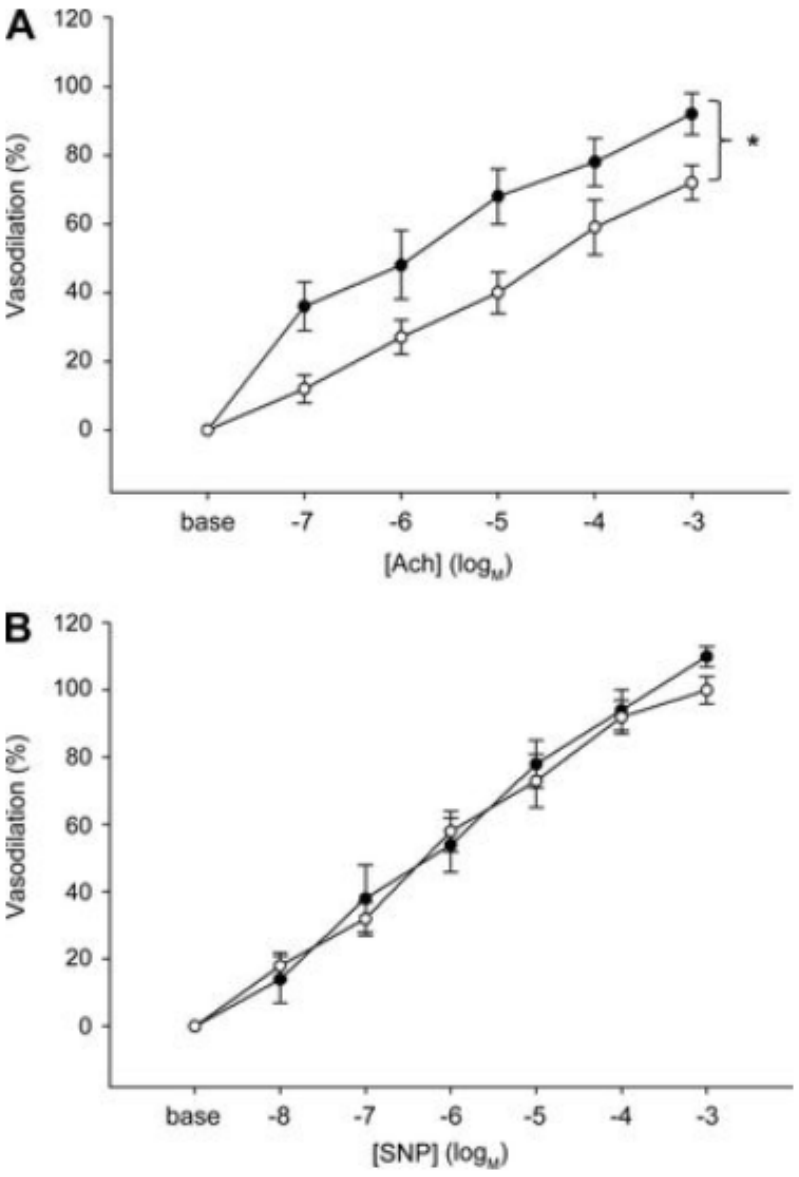

Fig. 4. SMFA endothelium-dependent vasodilation induced by increasing doses of ACh (*A*) and endothelium-independent vasodilation induced by increasing doses of SNP (*B*) in young and old subjects. Data are expressed as means ± SE; $n = 12$ young and 12 old subjects. *Significant difference between young and old subjects, $P < 0.05$.

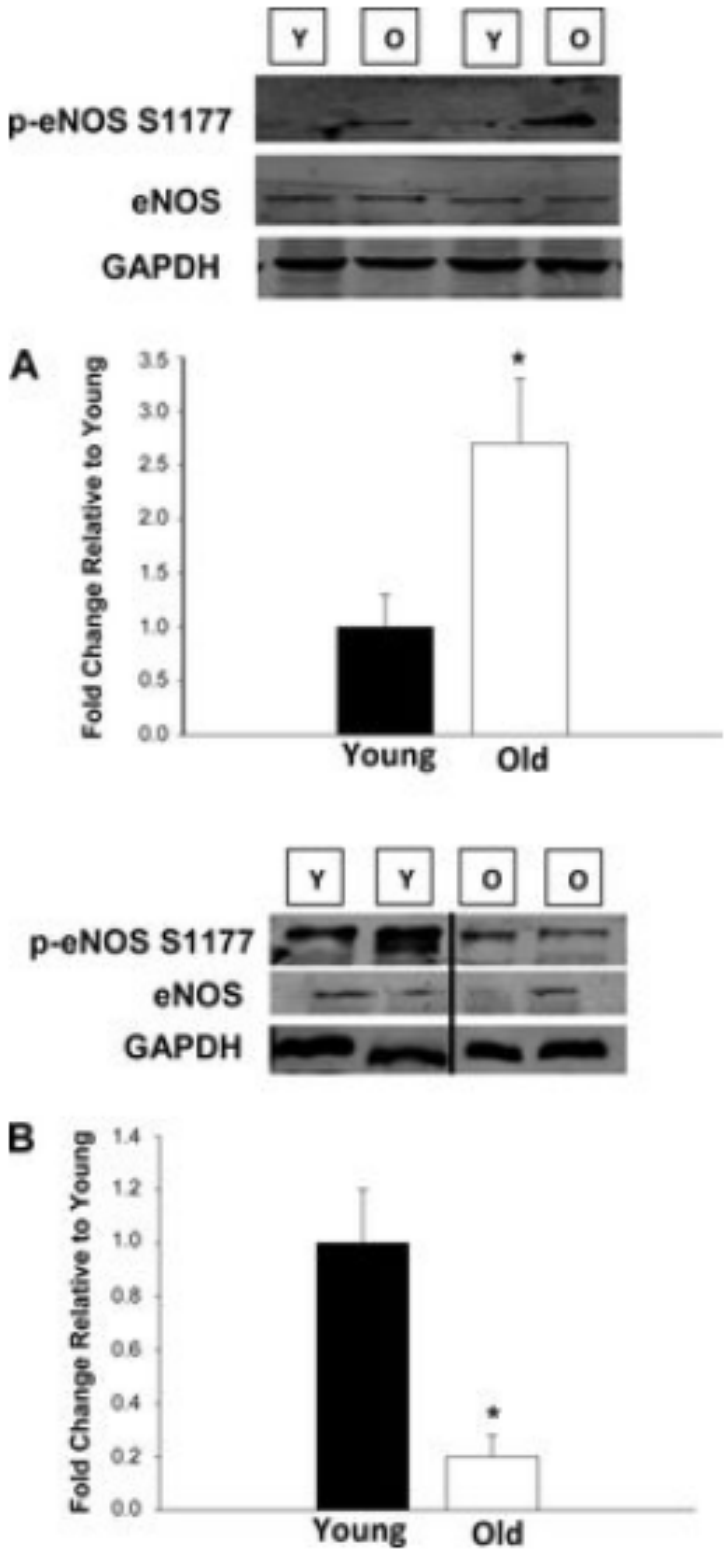

Fig. 5. Total endothelial NOS (eNOS) and phosphorylated (p-)eNOS at Ser^{1177} in SMFAs of young (y) and old (o) subjects under basal conditions (*A*) and changes induced by 6 min of flow inducing a shear rate of ~500 s^{-1} (*B*). Data are expressed as means ± SE; n = 8 young and 8 old subjects. *Significantly different from young subjects, $P < 0.05$.

Free radicals.

The baseline EPR spectroscopy signal for the CMH adduct, an index of superoxide concentration, was greater in the old group compared with the young group (old group: 3.9 ± 1.0 AUC/mg and young group: 1.7 ± 0.1 AUC/mg, $P < 0.05$; Fig. 6).

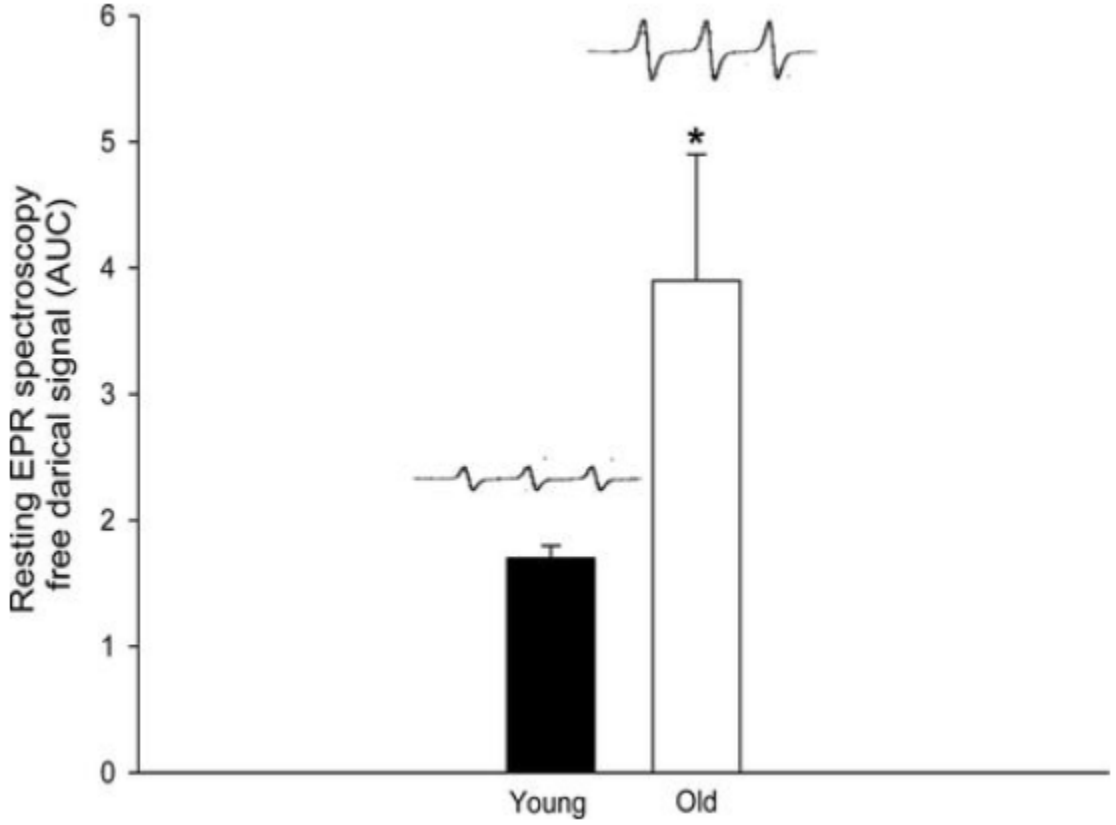

Fig. 6. Basal superoxide level, as assessed by 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine adduct, in young and old SMFAs assessed by electron paramagnetic resonance (EPR) spectroscopy. The EPR signal is expressed as the area under the curve (AUC; in arbitrary units). Representative spectroscopy signals are inlayed. Data are expressed as means ± SE; $n = 8$ young and 8 old subjects. *Significantly different from young subjects, $P < 0.05$.

DISCUSSION

Although aging is associated with attenuated skeletal muscle blood flow and SMFAs have been recognized to play a regulatory role in the vasculature, little is known about the impact of age on the vasodilatory capacity of human SMFAs. In this regard,

there are several interesting and novel findings of this study. First, these data both support and extend the inference that human SMFAs are likely regulatory in nature. Specifically, SMFAs are capable of significantly increasing vessel diameter in response to both increased shear stress and pharmacological vasodilators, the magnitude of which, in vivo, would likely result in significant changes in vascular conductance. Second, the magnitude of SMFA endothelium-mediated vasodilatory capacity, as assessed by the vasodilatory response to shear stress and ACh, is significantly attenuated with advancing age. In contrast, aging did not impact smooth muscle function. Third, in agreement with an age-related decrement in endothelium-mediated vascular function, shear-induced SMFA vasodilation kinetics are significantly attenuated with age. Finally, providing mechanistic insights into the age-related attenuation in SMFA vasodilatory function, there was evidence of attenuated shear-induced eNOS phosphorylation with age and greater levels of superoxide, suggestive of a role for reduced NO bioavailability. Given the potential impact of SMFAs in the regulation of blood flow during exercise, these findings may explain, at least in part, the attenuated perfusion of skeletal muscle with advancing age, which may contribute to exercise intolerance in the elderly.

Implication of attenuated SMFA vasodilation and vasodilation kinetics with age.

The regulation of O_2 delivery (and, therefore, blood flow at rest and during exercise) is an important component of homeostasis in both humans and animals (7, 21). In animals, it has been well documented that, compared with their younger counterparts, older animals exhibit an attenuated maximum endothelium-dependent

vasodilatory capacity in skeletal muscle arterioles, which results in an impaired O_2 delivery to working muscles (32, 33). Furthermore, Behnke et al. (2, 4), using an isolated vessel approach, revealed an age-related impairment in vasodilation kinetics. This would likely result in a temporal mismatch between O_2 delivery and O_2 consumption in skeletal muscle during the rest to exercise transition, ultimately resulting in a low microvascular PO_2 with advancing age (2, 3). Although this age-related transient attenuation in microvascular O_2 availability has been documented to be a consequence of delayed blood flow to the muscle at the onset of contraction in animals (2), to our knowledge, until now, there had not even been a single study that examined the effect of age on human SMFA vasodilation kinetics, which may have the same downstream consequences. Indeed, this appears to be the first study demonstrating a reduced magnitude of vasodilation and vasodilation kinetics in human SMFAs as a consequence of age (Figs. 1– 4), and the impact on muscle O_2 availability still needs to be determined.

Interestingly, at the onset of flow, the vasodilatory response of the current SMFAs, specifically the time to reach the peak diameter, was similar to previous studies that examined other human vessels (6, 29) but was significantly slower (~200 s) compared with the animal study performed by Behnke et al. (8 –23 s) (2). T was also significantly slower in human SMFAs compared with animal arterioles (2). However, both the slower T and time to reach the peak shear-induced vasodilatory response in human SMFAs may be due to the larger size of these vessels compared with arterioles of much smaller animals (34). Another clear distinction between these vessels is location, with SMFAs being external to the muscle, whereas arterioles are internal. Thus, each may exhibit differing vasodilatory characteristics as a consequence of being

a part of differing vascular beds (34). Of note, however, and in agreement with our previous study (21), but certainly not a focus of the current work, there was no evidence of different SMFA vasoactive responses as a consequence of anatomic location (i.e., axillary and inguinal regions). Interestingly, this implies that SMFAs of similar size from upper and lower limbs perform similarly in terms of vasodilation despite somewhat different origins and subsequently potentially quite different intravascular pressures in vivo.

Although it is currently not possible to quantify the exact physiological shear forces experienced by SMFAs in humans, based on studies in animals, Lipowsky et al. (26) suggested that an acceptable physiological shear rate for human arterioles would be in the 250-1,500 s^{-1} range. Furthermore, Fisslthaler et al. (15) demonstrated that the induction of a shear stress of 12 dyn/cm^2, equivalent to a shear rate of 200 s^{-1}, can significantly increase eNOS activation in cultured human umbilical vein endothelial cells. It should be noted that the procedures in the present study resulted in a shear rate of ~500 s^{-1}, which stimulated significant eNOS activation and SMFA dilation. Interestingly, the change of pressure of ~30 mmHg used in the present study yielded a significantly greater vasdilatory response than the change of pressure of ~15 mmHg, whereas greater increases in the pressure difference (changes of ~45 and ~60 mmHg) assessed in pilot studies resulted in more subtle increases in vasodilation. These data could be interpreted to indicate that a shear rate of ~500 s^{-1} is a physio- logically relevant stimulus for human SMFAs, and advancing age blunts the vasodilatory response to such a level of shear.

Potential mechanisms responsible for the age-related attenuation in SMFA vasodilation.

Previous animal studies have suggested that there is an attenuated vascular conductance and a limited increase in red blood cell flux over time in muscle during dynamic exercise with advancing age (8, 17), indicative of compromised peripheral circulatory function (2, 8, 18). These prior studies and other work focused on the impact of age on the microvasculature during exercise in animals have revealed slower endothelium-dependent vasodilator dynamics in skeletal muscle arterioles due to the reduced NO bioavailability in the old (2, 18, 33). In agreement with these studies, the present results extend these findings to human SMFAs, revealing that aging reduces flow-induced (Figs. 1 and 2) and ACh-induced (Figs. 3 and 4) vasodilation, both indicators of endothelium-dependent dysfunction. Supportive of an important role of NO in the flow-induced vasodilation assessed in this study was the observation that the percentage of vasodilation in response to both increased shear stress and ACh was significantly attenuated in both young and old groups in the presence of L-NMMA, a NOS inhibitor. Furthermore, and of importance with regard to the impact of age, there was a significantly greater reduction in the percentage of vasodilation in the presence of L-NMMA in the young group compared with the old group (Fig. 3). This finding supports the supposition that the contribution of NO to the SMFA percentage of vasodilation, in response to both increased shear stress and ACh, is greater in the young than in the old, providing evidence that aging attenuates NO bioavailability in these vessels, which is well aligned with previous studies in arterioles (2, 33).

Furthermore, the present study revealed an attenuated in- crease in p-eNOS

Ser^{1177} to total eNOS protein expression, a well-described activation site on eNOS (45), in SMFAs from old subjects in response to an increase in shear stress, providing a mechanistic basis for the age-related attenuation in both the magnitude of vasodilation and vasodilation kinetics (Fig. 5*B*). Interestingly, SMFAs from old subjects also exhibited significantly greater basal p-eNOS Ser^{1177} to total eNOS protein expression compared with young SMFAs (Fig. 5*A*), which contrasts with the predominant findings in the microvasculature of animals (44, 45, 52). However, these findings are well aligned with previous assessments of the vasculature in hu- mans that have documented a greater activation of p-eNOS Ser^{1177} with age at rest, which was interpreted as an attempt to compensate for low NO bioavailability (12, 40). Overall, these findings suggest that baseline eNOS activation is increased in old SMFAs to compensate for the attenuated NO bioavailability; however, old SMFAs appear less capable of activating eNOS in response to an increased shear stimulus compared with young SMFAs. This observation is in line with previous work that examined upstream mechanisms and found less capacity to phosphorylate eNOS at Ser^{1177} in older adults than their younger counterparts (46). Additionally, the EC_{50} for ACh, indicative of the sensitivity of muscarinic receptors in the endothelium, was reduced with age, which may also play a role in the attenuated pharmacologically induced vasodilation kinetics in the old.

It has been suggested that an increase in the production of ROS and/or limited antioxidant capacity with advancing age attenuates endothelial function in humans and animals (1, 33, 51). Interestingly, albeit in the basal state, the present study provides evidence of greater superoxide levels (CMH adduct) in old compared with young SMFAs, as directly assessed by EPR spectroscopy (Fig. 6). Functionally, the reaction of

NO with superoxide, to produce peroxynitrite (40), plays a major role in lowering NO bioavailability, not only directly but also indirectly, as peroxynitrite can uncouple eNOS. Indeed, Liu et al. (29) documented that shear stress-induced ROS production in human coronary arteries decreases NO bioavailability, and others have documented that ROS impair NO-mediated vasodilation in peripheral arteries with age (14, 28, 29, 32).

Interestingly, recent studies have suggested that in the presence of blunted NO-mediated dilation, due to decreased NO bioavailability, compensatory vasodilatory mechanisms are favored in human arteries (6, 27, 31, 36). These elegant studies revealed that an attenuated vasodilatory capacity, due to attenuated NO bioavailability with advancing age and/or in disease, can be preserved by compensatory mechanisms such as H_2O_2- mediated vasodilation. H_2O_2 has been established as a potent vasoactive mediator released from the endothelium in response to increased shear stress in both human and animal arteries and can preserve vasodilatory function in the face of attenuated NO-mediated dilation (6, 27, 31, 36). Such compensatory mechanisms may have been in play in the old vessels, potentially being involved in the observed attenuated magnitude of NO-mediated vasodilation and the slowed vasodilation kinetics in response to increased shear stress in SMFAs from old subjects (Fig. 2). Specifically, a compensatory mechanism, such as H_2O_2-mediated vasodilation, may not be as effective as NO in terms of either vasodilatory capacity or the speed of vasodilation; however, such speculation needs further studies to confirm this notion. Regardless, the present study confirms that aging attenuates human SMFA vasodilatory capacity as well as vasodilation kinetics of human SMFAs and provides evidence that, mechanistically, this is likely due to decreased NO bioavailability.

The regulatory function of SMFAs.

Previous animal studies have suggested that SMFAs are capable of altering muscle blood flow by manipulating vascular conductance in accordance with metabolic requirements (23, 42). This vasoconstriction and vasodilation, previously documented in vitro, would likely translate into significant alterations in basal vascular resistance in vivo (41– 43). Recent work by our group has provided translational evidence of the regulatory capability of human SMFAs, elicited by pharmacological stimulation, by documenting significant changes in calculated vascular conductance (21). In addition to these previous studies, and similar to that observed in a rodent model (22), the present study confirms and extends this evidence of the regulatory potential of human SMFAs by documenting vasodilation in response to a more physiologically relevant stimulus, an increase in shear stress. These findings suggest that SMFAs may be an important factor in regulating local blood flow at rest and during challenges to homeostasis, such as exercise and orthostasis, in humans. Importantly, this regulatory potential appears to be compromised with advancing age, and this may have significant consequences in terms of skeletal muscle blood flow in the elderly.

Experimental considerations.

The fundamental intent of this study was to examine the impact of aging on vasodilatory capacity, including vasodilation kinetics in response to flow, in human SMFAs. As we used an in vitro approach to achieve this goal, it is now unclear whether the relatively slow kinetics are a consequence of this approach or represent the scenario in vivo. Indeed, the observed kinetics appear rather slow to contribute usefully

to the regulation of blood flow at the onset of exercise, for example. Factors that may have played a role in this potential slowing of vasodilation kinetics in vitro in- clude differing external pressures around the vessels, the lack of a muscle pump and mechanical deformation associated with the initiation of muscle contraction, and the absence of neural signals. Additionally, in this regard, the use of a single flow rate to induce shear stress-mediated vasodilation and not a dose response may have influenced the present findings. However, of note, despite these uncertainties, this study clearly documented an age-related attenuation in SMFA vasodilation kinetics (Fig. 2). Additionally, recognizing that physical activity can alter vascular function, a limitation of the present study is the lack of a physical activity assessment of the subjects. Therefore, as with many studies of aging, these findings may have been affected by differences in physical activity with advancing age, and this potential warrants further investigations with a focus on physical activity.

Summary.

The present study used both a physiologically relevant flow-induced increase in shear stress and a pharmacologically induced vasodilation to assess the impact of aging on vasodilatory capacity and vasodilation kinetics in human SMFAs. We have identified that the endothelium-dependent vasodilatory capacity and vasodilation kinetics, but not endothelium-independent vasodilatory capacity and smooth muscle function, of human SMFAs were blunted with age. This attenuated endothelium-dependent vascular function seems to be explained by attenuated eNOS activation and subsequently NO bioavailability and may be a consequence of elevated free radicals

with age. Given the likely regulatory role of human SMFAs in skeletal muscle blood flow, these findings may explain, at least in part, the attenuated perfusion of skeletal muscle often exhibited with advancing age that may contribute to exercise intolerance in the elderly.

ACKNOWLEDGMENTS

The authors thank the volunteers for participation as well as the surgical staff at Huntsman Cancer Hospital.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant PO1-HL-091830 (to R. S. Richardson) and Veterans Affairs Merit Grant E6910R (to R. S. Richardson).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.-Y.P., J.R.G., and R.S.R. conception and design of research; S.-Y.P., S.J.I., R.H.A., J.R.H., V.R.R., G.L., and L.P.B. performed experiments; S.-Y.P., G.L., L.P.B., and J.D.S. analyzed data; S.-Y.P. and S.J.I. interpreted results of experiments; S.-Y.P., R.H.A., and R.S.R. drafted manuscript; S.-Y.P., J.R.H., J.D.S., and R.S.R. edited and revised manuscript; S.-Y.P. and R.S.R. approved final version

of manuscript; R.S.R. prepared figures.

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