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Recognizing the age-related decline in skeletal muscle feed artery (SMFA) vasodilatory function, this study examined the link between vasodilatory and mitochondrial respiratory function in the human vasculature. Twenty-four SMFAs were harvested from young (35 ± 6 yr, *n* = 9) and old (71 ± 9 yr, *n* = 15) subjects. Vasodilation in SMFAs was assessed, by pressure myography, in response to flow-induced shear stress, acetylcholine (ACh), and sodium nitroprusside (SNP) while mitochondrial respiration was measured, by respirometry, in permeabilized SMFAs. Endothelium-dependent vasodilation was significantly attenuated in the old, induced by both flow (young: 92 ± 3, old: 45 ± 4%) and ACh (young: 92 ± 3, old: 54 ± 5%), with no significant difference in endothelium-independent vasodilation. Complex I and I + II state 3 respiration was significantly lower in the old (CI young: 10.1 ± 0.8, old: 7.0 ± 0.4 pmol·s^{-1}·mg^{-1}; CI + II young: 12.3 ± 0.6, old: 7.6 ± 0.4 pmol·s^{-1}·mg^{-1}). The respiratory control ratio (RCR) was also significantly attenuated in the old (young: 2.2 ± 0.1, old: 1.1

± 0.1). Furthermore, state 3 (CI + II) and 4 respiration, as well as RCR, were significantly correlated (r = 0.49 – 0.86) with endothelium-dependent, but not endothelium-independent, function. Finally, the direct intervention with mitochondrial-targeted antioxidant (MitoQ) significantly improved endothelium-dependent vasodilation in the old but not in the young. Thus, the age-related decline in vasodilatory function is linked to attenuated vascular mitochondrial respiratory function, likely by augmented free radicals.

NEW & NOTEWORTHY In human skeletal muscle feed arteries, the well-recognized age-related fall in endothelium-dependent vasodilatory function is strongly linked to a concomitant fall in vascular mitochondrial respiratory function. The direct intervention with the mitochondrial-targeted antioxidant restored vasodilatory function in the old but not in the young, supporting the concept that exacerbated mitochondrial-derived free radical production is linked to age-related vasodilatory dysfunction. Age-related vasodilatory dysfunction in humans is linked to attenuated vascular mitochondrial respiratory function, likely a consequence of augmented free radical production.

Keywords: mitochondrial function; mitochondrial-targeted antioxidant; vasodilatory function vascular

INTRODUCTION

Advancing age is associated with attenuated blood flow and oxygen delivery to skeletal muscle (3, 35). This age-related alteration in blood flow distribution appears to be, somewhat, due to diminished vasodilatory function with advancing age, mediated by nitric oxide (NO) bioavailability in the resistance vessels of skeletal muscle (2, 10, 38, 39). Skeletal muscle feed arteries (SMFAs) are inlets to the skeletal muscle bed and are upstream of the arterioles. Because of this anatomical location, in both animals and humans, SMFAs have been suggested to regulate skeletal muscle perfusion (19, 37). Recently, it has been documented that SMFAs, collected from human subjects and studied in vitro, play a potential role in regulating vascular resistance and subsequently

blood flow to skeletal muscle (19). Furthermore, in terms of aging, our group recently reported that the endothelium-dependent vasodilation and NO bioavailability in SMFAs are attenuated in the elderly, suggesting that impaired SMFA vasodilatory function may play a role in the attenuated blood flow noted with advancing age (31). How- ever, the exact fundamental mechanism that contributes to this age-related impairment in SMFA function is unknown.

A typical characteristic of aging, even across diverse tissue, such as skeletal muscle, heart, brain, and liver, is a progressive dysregulation of mitochondria, essential intracellular organelles producing energy for critical cellular processes (15, 25, 27, 33). The dominant pathological characteristics of mitochondria with advancing age are the degradation of mitochondrial function and increased mitochondrial-derived free radical production during oxidative phosphorylation. Indeed, age-related structural and functional alterations in the mitochondrial respiratory chain lead to severe electron leak that repeatedly increases free radical levels with advancing age (13, 24), leading to a vicious positive feedback cycle that exacerbates macromolecular damage. At the cellular level, structure and function are also often compromised by this diminished energy source, also resulting in greater oxidative stress, which leads to systemic damage and dysfunction.

Although the exact role of mitochondrial respiration in the vasculature is not clear, there are several studies demonstrating the impact of mitochondrial-derived free radicals on vascular function. For example, in aged rodent arteries, scavenging mitochondrial-derived free radicals reduces the risk of vascular inflammation (42) and restores vascular function to, almost, the same level of that of the young (14). Furthermore, in human SMFAs, our group recently documented that, with aging, vascular mitochondrial respiratory function declines and, per mitochondrion, there appear to be greater mitochondrial-de- rived free radicals (29). However, it should be noted that our previous study was observational in nature and focused on chronological age and not vascular function, per se. Nonetheless, these studies support the premise that attenuated vascular function with advancing age may be associated with mitochondrial-derived free radicals, which are directly produced by the mitochondrial respiratory chain. However, unlike the relatively well-understood link between attenuated

mitochondrial respiratory function and aging in other tissue, little is known about the interdependence between vasodilatory function and vascular mitochondrial respiratory function in the vasculature with advancing age.

Consequently, with the intent to evaluate the potential age-related interdependence of vasodilatory and metabolic function in the vasculature of human skeletal muscle, this study used pressure myography and respirometry to assess SMFA vasodilatory and vascular mitochondrial respiratory function, respectively, in young and old subjects. Given the recognized functional alterations in other tissue with advancing age, due to attenuated mitochondrial respiration, we tested the hypothesis that human SMFAs exhibit age-related vascular dysfunction and this is linked to attenuated vascular mitochondrial respiratory function. Furthermore, we also hypothesized that the interdependence between vasodilatory and vascular mitochondrial respiratory function is a consequence of mitochondrial free radical production.

METHODS

Subjects. A heterogeneous group of 24 subjects (9 young, <40 yr and 15 old, >60 yr) were studied in the main comprehensive investigation. Another 22 subjects were pooled with data from the main investigation to provide more complete spectrum of age and bolster subject number for the correlational analyses. An additional 22 subjects (9 young and 13 old) were enrolled to perform an interventional investigation to provide mechanistic insight into the findings of the present study (Table 1). Of note, data for all subject groups were collected and analyzed with the same methods and protocols. All subjects agreed to have their vessels harvested during surgery and used in this study. None of the subjects was taking medications recognized to alter vascular and mitochondrial function, and each was predominantly free from overt cardiovascular disease (Table 1). All protocols were approved by the Institutional Review Boards of the University of Utah and the Salt Lake City Veteran's Affairs Medical Center, and written informed consent was obtained by all subjects before surgery.

The origin of the skeletal muscle feed arteries. As previously reported (17, 18), human skeletal muscle feed arteries (~7 mg/wet wt), supplying the axillary (e.g., serratus anterior or latissimus dorsi) and inguinal regions (e.g., quadriceps femoris or hip

adductors), were obtained during melanoma-related node dissection surgery. All vessels were excised, immediately stored in precooled physiological saline solution (PSS), and brought to the laboratory within 15 min of harvesting for analysis. It is acknowledged that, because of scarcity of the SMFAs used in this work, 6 out of 23 SMFAs from young subjects (26%) and 5 out of 33 SMFAs from old subjects (15%) in the current study have been used in a previous publication (29).

Vasodilatory function assessment. Adipose and connective tissue around the SMFAs was removed under a microscope (SZX10; Olympus, Center Valley, PA) in 4°C PSS with which they were also thoroughly rinsed [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, 3.0 MOPS, and 10 g/L of BSA at pH 7.4]. With the use of a pressure myograph system (110p; DMT Systems, Aarhus, Denmark), the SMFAs were cannulated at both ends with micropipette tips in the chamber. SMFAs were preincubated for 1 h in 37°C PSS. The vessel outer diameters were then recorded using 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 0.2.0). Arteries free from fluid leaks were then used to assess vasodilatory function. Following the preconstriction with phenylephrine (PE, 10^{-6} to 10^{-4} M; Sigma-Aldrich) to ~70% of the maximum PE response, the vasodilatory function was assessed in response to the following three stimuli: *1*) an endothelium-dependent flow-induced shear stress dose response. 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 mean intraluminal pressure. Three pressure differences of 15, 30, and 40 mmHg, which yielded approximate flow rates of 15, 30, and 45 μl/min, assessed with a flowmeter (162FM, DMT Systems), were used for the flow experiments; *2*) an endothelium-dependent acetylcholine (ACh) dose response (10^{-7} to 10^{-3} M; Sigma-Aldrich); and *3*) an endothelium-independent sodium nitroprusside (SNP) dose response (10^{-8} to 10^{-3} M; Sigma-Aldrich). Each of these responses was assessed with the vessel pressurized at 60 mmHg, as previously performed (19, 31, 32, 45).

Table 1. ***Subject characteristics***

	Main Investigation		Additional Subjects for the Pooled Correlation Analysis (Fig. 7)			Total Pooled Subjects (n=46)	Interventional Investigation (Fig. 6)	
	Young (n=9)	Old (n=15)	Young (n=5)	Middle (n = 12)	Old (n=5)		Young (n=9)	Old (n=13)
Age, yr	34 ±6*	71±9	30±6	54±5	63±4	54±17	30±5*	77±7
Sex (male/female), *n*	3/6	12/3	3/2	8/4	3/2	29/17	5/4	8/5
Height, cm	171±10	171±7	168±9	174±7	172±4.2	172±8	176±15	164±13
Body mass, kg	80±23	89±21	89±14	91±19	83±12.9	87±19	75±14	80±11
BMI, kg/m^2	28±7	30±7	30±4	30±7	27±3	29±6	22±7	28±8
Systolic blood pressure, mmHg	118±11	131±18	132±12	127±11	132±14	128±14	117±8	129±9
Diastolic blood pressure, mmHg	69±16	76±11	81±12	78±10	78±9	76±12	75±5	83±8
Medication history, users/*n*								
Cardiovascular								
Statins	0/9	1/15	0/5	2/12	0/5	3/46	0/9	0/13
Cholesterol medication	1/9	1/15	0/5	1/12	1/5	4/46	0/9	0/13
Anticoagulant	0/9	1/15	0/5	0/12	1/5	2/46	0/9	0/13
Metabolic								
Antidiabetic medication	0/9	1/15	0/5	0/12	0/5	1/46	0/9	1/13
Analgesic								
NSADs	2/9	2/15	0/5	2/12	2/5	8/46	0/9	0/13
Hormonal								
Melatonin	0/9	1/15	0/5	0/12	1/5	2/46	0/9	0/13
Thyroid	0/9	1/15	0/5	1/12	0/5	2/46	0/9	0/13

Data are expressed as means ± SD, sex (male/female), or no. of subjects (of the total number; *n*). BMI, body mass index; NSAIDS, nonsteroidal anti-inflammatory drugs. *Significant difference young and old

Percentage vasodilation calculations. Percentage vasodilation was used to account for baseline differences in vessel diameter and calculated using the following equation: (D_{Dose}– D_P ⁄ D_I– D_P) X 100, where D_{Dose} is the recorded diameter as consequence of a given treatment (i.e., drug dose or flow rate), D_P is the diameter recorded after the addition of the vasoconstrictor (i.e., PE), and D_I is the diameter recorded immediately before the addition of the vasoconstrictor (initial diameter).

Vascular mitochondrial respiration assessment. Vessel samples were cleaned as for the vasodilatory function assessment and then prepared and permeabilized for mitochondrial respiration analysis, as previously described by Park et al. (30). Briefly, vessels were stored in ice-cold biopsy preservation fluid (BIOPS) [containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 DTT, 50 K-MES, 20 imidazole, 20 taurine, 5.77 Na_2ATP, and 15 phosphocreatine, pH 7.1 at 4°C] for 30 min before the permeabilization procedure (20). BIOPS-immersed vessels were carefully separated with fine-tip for- ceps and subsequently bathed and shaken in a BIOPS with saponin

solution (50 mg/ml) for 40 min. Following saponin treatment, vessels were rinsed two times in MIR05 [containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 DTT, 20 imidazole, 5.77 ATP, 15 phosphocreatine, 50 K-MES, and 20 taurine, pH 7.0] for 20 min.

Vessel samples were then placed in the temperature-controlled Clark-type high-resolution Oxygraph respirometer (Hansatech, Kings Lynn, UK) in 2 ml MIR05 solution and were continuously stirred at 37°C. After the vessel samples were allowed to equilibrate for 10 min, mitochondrial respiratory function was assessed using the protocol described in Table 2. To measure the function of each mitochondrial complex, O_2 consumption was assessed with the addition of a series of respiratory substrates and inhibitors in the following order and final concentrations in the chamber: glutamate-malate (2, 10 mM), ADP (5 mM), succinate (10 mM), rotenone (0.5 μ,M), cytochrome c (10 μ,M), antimycin A (2.5 μ,M), and oligomycin (2 g/ml). This allowed the determination of *1*) complex I (CI) state 3 respiration, the ADP-activated state of oxidative phosphorylation, assessed in the presence of glutamate, malate, and ADP, *2*) complex I + II (CI + II) state 3 respiration, assessed in the presence of glutamate, malate, ADP, and succinate, *3*) complex II (CII) state 3 respiration, assessed in the presence of glutamate, malate, ADP, succinate, and rotenone, *4*) mitochondrial membrane integrity, assessed in the presence of cytochrome c, and *5*) state 4 respiration, assessed by blocking ATP synthase (oligomycin). In each condition, respiration rate was re- corded for 3 min. The rate of O_2 consumption was measured as picomoles of O_2 per second and then expressed relative to vessel sample mass ($pmol \cdot s^{-1} \cdot mg\ wet\ wt^{-1}$). The respiratory control rate (RCR) was calculated by state 3/state 4 respiration.

Table 2. ***Mitochondrial respiration protocol***

Steps	Chemical	Site of Action	Respiration State
1	Malate (2mM), glutamate (10mM)	+Complex I	Complex I, state 3
	ADP (5mM)	+Complex V	
2	Succinate (10mM)	+Complex II	Complex I+II, state 3
3	Rotenone (0.5 μM)	-Complex I	Complex II, state 3
4	Cytochrome c (10 μM)	Test of membrane integrity	
5	Oligomycine (2 g/ml)	Complex V	State 4

Description of the protocol used to assess mitochondrial respiratory function, the site of action of each chemical introduced to the preparation (+substrate; -inhibitor), and the respiration state associated with each step. Note that each step took ~3 min before proceeding to the next step.

Mitochondrial-targeted antioxidant and vessel function assessment. Following preparation for the vasodilatory function assessments, as described above, the arteries were cannulated at both ends with micropipette tips and then preincubated for 1 h within the bath in either PSS, the control condition, or a commercially available mitochondrial-targeted antioxidant, MitoQ (10 μ,M; MitoQ Limited, Auckland, NZ). After the preincubation period, the vasodilatory function of the vessels was assessed, as described above.

Statistical analysis. For vascular function assessments, two-way repeated ANOVA was performed using SPSS (version 22; SPSS, Chicago, IL). If significance was detected, Tukey's post hoc test was used to identify the significant difference. For mitochondrial respiration assessments, a Student's *t* test was performed. Correlations between variables were assessed by Pearson product-moment correlation analyses. It should be noted that this line of best-fit approach only accounts for the variation in the dependent variables. Multiple-regression analyses and 3D graphing were performed using R Studio (version 1.1.443; Boston, MA) and R (version 3.4.4; The R Foundation, Vienna, Austria) with the gem and plotty packages. All data are expressed as means ± SE. Statistical significance was set at $P < 0.05$.

RESULTS

Vessel and subject characteristics. For the main investigation, 24 human SMFAs from either the inguinal or axial regions were successfully harvested in young (35 ± 6 yr, n =9) and old (71 ± 9 yr, n = 15) subjects. Baseline vessel diameter, after pressurization, was not significantly different between young (415.9 ± 92.1 μ,m) and old (428.3 ± 91.1 μ,m). Before mitochondrial respiration was assessed, each SMFA was trimmed and weighed to facilitate the normalization of respiration rate by wet weight and also to facilitate the active minimization of the variation in sample mass. Of note, in agreement with our previous observations, there was no evidence of a difference in vasodilatory function in terms of SMFA anatomic region of origin of the subject. Furthermore, there was no effect of sex on either mitochondrial respiration or vasodilatory function.

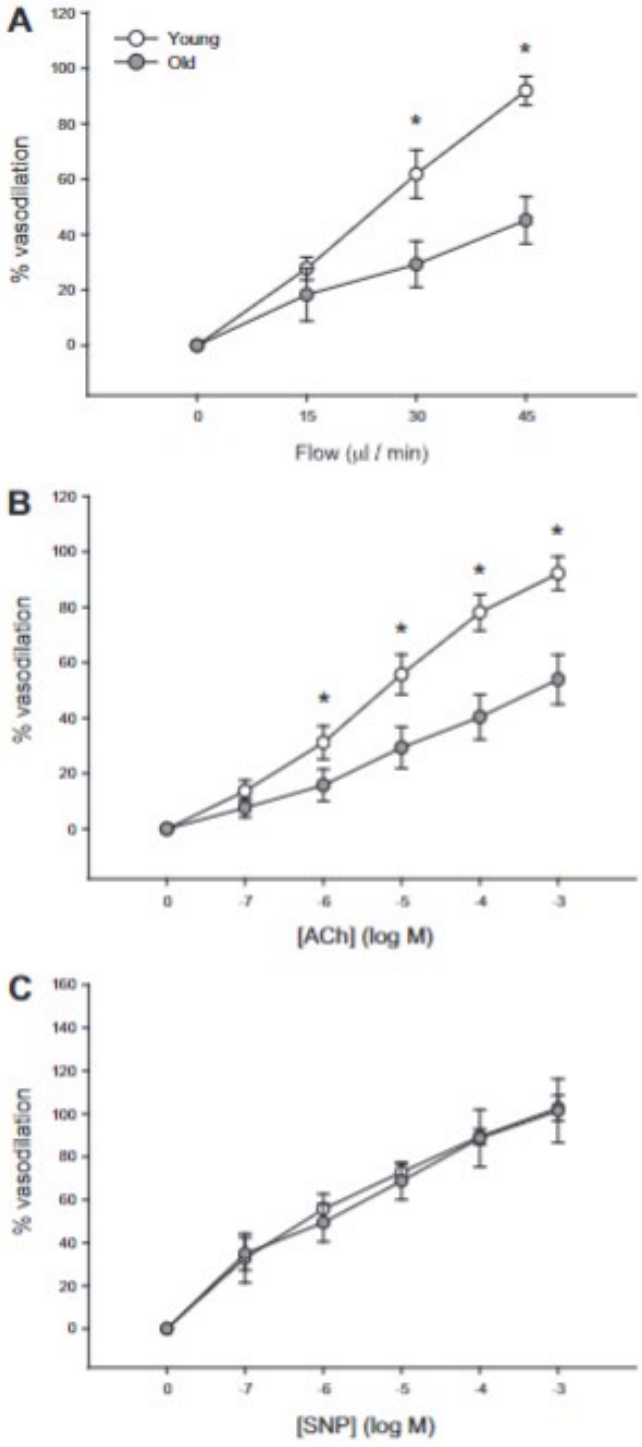

Fig. 1. The vasodilatory capacity of skeletal muscle feed arteries from young and old subjects. *A*: the endothelium-dependent vasodilation curve mediated by different flow rates (15, 30, and 45 µ,l/min) in 9 young (3 males and 6 females) and 15 old (12 males and 3 females) subjects. *B*: the endothelium-dependent acetylcholine (ACh) concentration response curve in 8 young (3 males and 5 females) and 13 old (10 males and 3 females) subjects. *C*: the endothelium- independent sodium nitroprusside (SNP) concentration response curve in 5 young (2 males and 3 females) and 8 old (6 males and 2 females) subjects. Brackets denote concentration. Data are expressed as means ± SE. Two-way repeated ANOVA was used. *Significant difference between young and old, $P < 0.05$.

Vasodilatory function with advancing age. The endothelium-dependent vasodilatory response to intraluminal flow was significantly attenuated in the old compared with the young at both 30 (young: 62 ± 4%, old: 29 ± 4; $P < 0.05$; Fig. 1*A*) and 45 (young: 92 ± 3%, old: 45 ± 4; $P < 0.05$; Fig. 1*A*) μ,l/min. In addition, SMFAs from the old exhibited a significantly attenuated endothelium-dependent vasodilatory response, com- pared with the young, across the majority of the ACh concentration response curve (CRC), with the largest difference being evident at the highest ACh concentration (10^{-3} M, young: 92 ± 3%, old: 54 ± 4; $P < 0.05$; Fig. 1*B*). In contrast, the endothelium-independent vasodilatory response to SNP (10^{-3} M) was not significantly different between the young and old across the whole CRC curve (Fig. 1*C*). Note, because of the loss of physiological responsiveness in some vessels through the complete process of the experiments (i.e., flow, ACh, and SNP), the number of samples differs across the various correlation analyses.

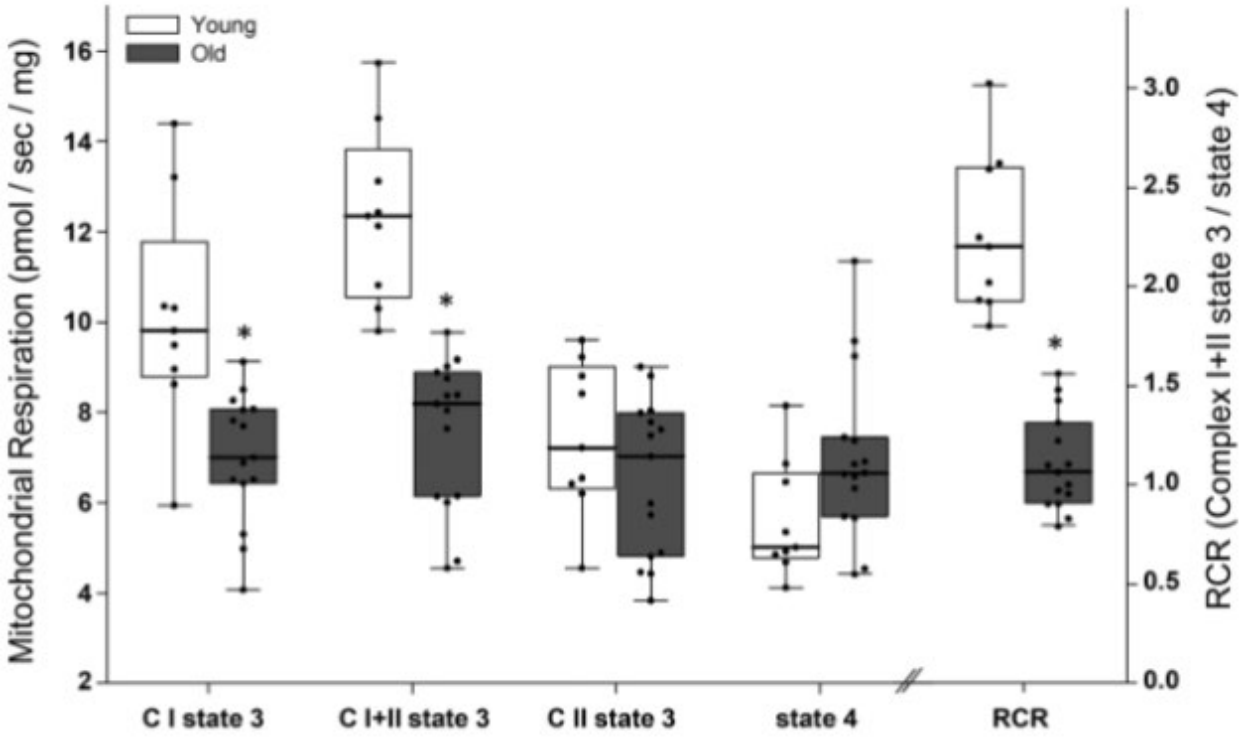

Fig. 2. The vascular mitochondrial respiratory function of skeletal muscle feed arteries from young and old subjects. Data are expressed with a box and whisker plot. The box indicates interquartile range from 25th to 75th percentiles, and a line within the box marks the median. Whiskers above and below the box indicate the maximum and minimum. RCR, respiratory control ratio. A Student's *t* test was used with 9 young (3 males and 6 females) and 15 old (12 males and 3 females) subjects. *Significant difference between young and old, $P < 0.05$.

Mitochondrial respiratory function with advancing age. As illustrated in Fig. 2, CI- and CI + II-driven state 3 respiration exhibited a significant difference between young and old. Specifically, oxidative phosphorylating respiration was significantly attenuated in the old compared with the young (CI young: 10.1 ± 0.8, old: 7.0 ± 0.4 pmol·s^{-1}·mg^{-1}; CI + II young: 12.3 ± 0.6, old: 7.6 ± 0.4 pmol·s^{-1}·mg^{-1}; $P < 0.05$; Fig. 2). State 4 respiration, an index of proton leak representing nonphosphorylating respiration, was not significantly different between groups but tended to be greater in old compared with young subjects (young: 5.6 ± 0.4, old: 7.0 ± 0.5 pmol·s^{-1}·mg^{-1}, $P = 0.06$; Fig. 2). In addition, the RCR, net oxidative coupling rate, calculated as state 3 (CI + II)/state 4 respiration, was significantly lower in the old compared with the young (young: 2.2 ± 0.1, old: 1.1 ± 0.06; $P < 0.05$; Fig. 2).

Relationship between oxidative phosphorylating respiration and vasodilatory function. The assessment of respiratory function and endothelium-dependent and -independent vasodilatory function in SMFAs from young and old subjects revealed a significant positive correlation between oxidative phosphorylating (CI + II) respiration and endothelium-dependent vasodilation, as assessed by both flow-mediated and ACh-dependent vasodilation ($r = 0.76$ and 0.7 respectively; $P < 0.05$; Fig. 3, *A* and *B*). In contrast, state 3 (CI + II) respiration did not exhibit a significant relationship with endothelium-independent vasodilation, as assessed by the SNP-induced response (Fig. 3*C*). Of note, when assessed separately, complex I-driven state 3 respiration was significantly correlated with ACh- and flow-induced vasodilation ($r = 0.53$ and 0.51 respectively; $P < 0.05$), whereas complex II-driven state 3 respiration was not.

Relationship between nonphosphorylating respiration and vasodilatory function. Although not reaching statistical significance ($P = 0.06$), endothelium-dependent flow-mediated vasodilatory function tended to decrease with evidence of greater nonphosphorylating state 4 respiration ($r = -0.35$; Fig. 4*A*). Furthermore, state 4 respiration exhibited a significant negative correlation with endothelium-dependent ACh-induced vasodilation ($r = -0.49$, $P < 0.05$; Fig. 4*B*). In contrast, there was no evidence of a correlation between state 4 respiration and endothelium-independent SNP-induced vasodilation (Fig. 4*C*).

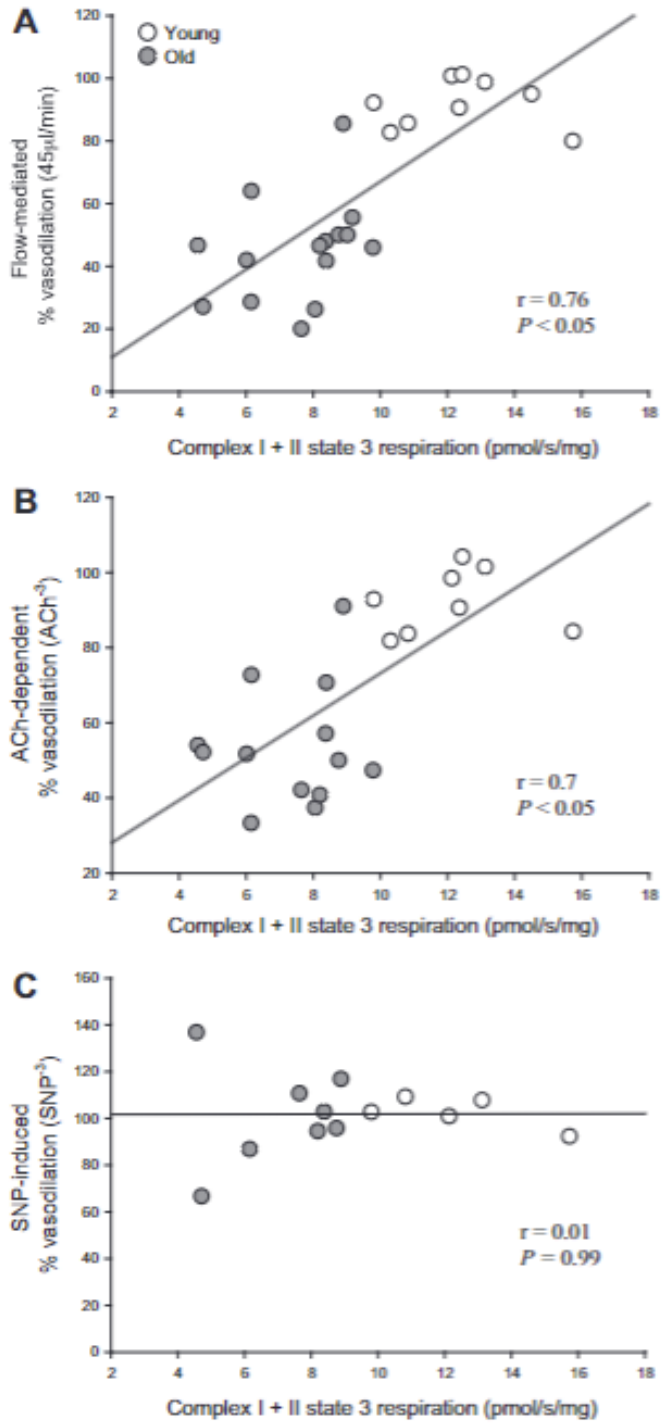

Fig. 3. The relationship between oxidative phosphorylating respiration and vasodilatory capacity. *A*: the correlation between state 3 (complex I + II) respiration and the greatest flow-mediated vasodilatory response (45 µ,l/min) in 9 young (3 males and 6 females) and 15 old (12 males and 3 females) subjects. *B*: the correlation between state 3 (complex I + II) respiration and the greatest ACh-dependent vasodilation (10^{-3}) in 8 young (3 males and 5 females) and 13 old (10 males and 3 females) subjects. *C*: the correlation between state 3 (complex I + II) respiration and the greatest sodium nitroprusside (SNP)-induced vasodilation (10^{-3}) in 5 young (2 males and 3 females) and 8 old (6 males and 2 females) subjects.

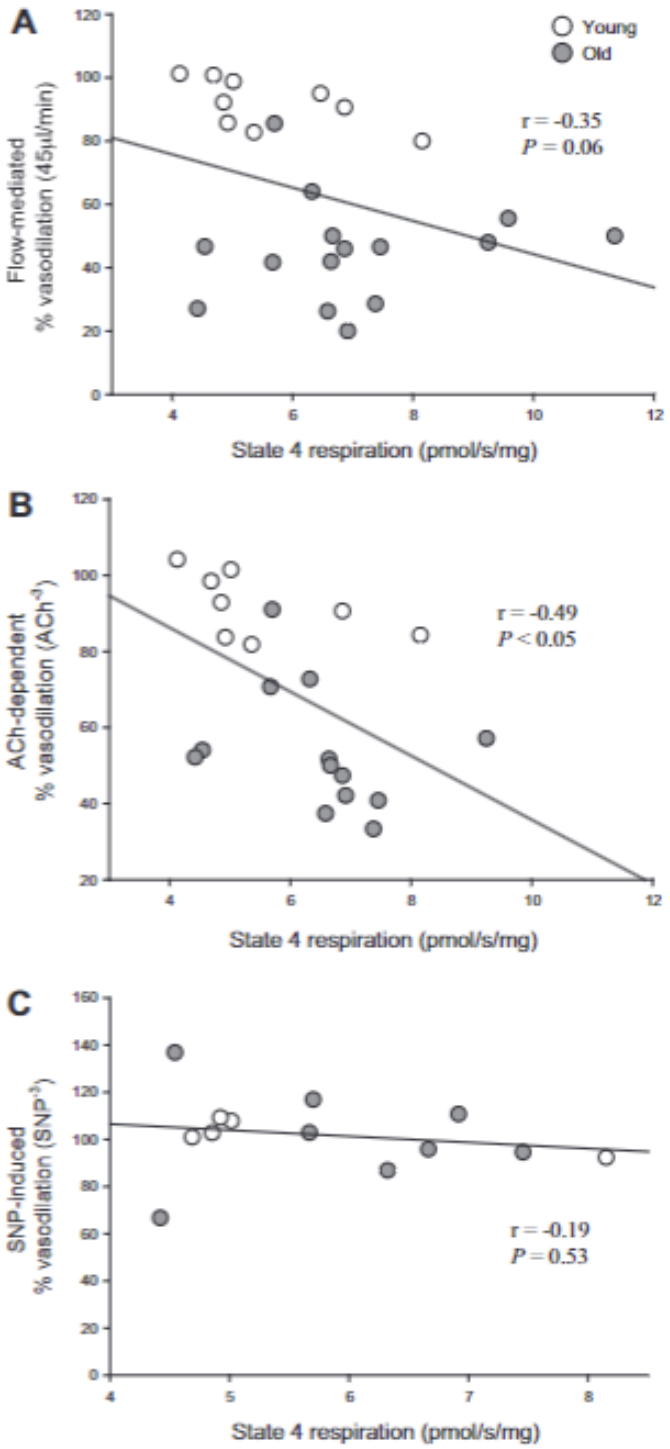

Fig. 4. The relationship between nonphosphorylating respiration and vasodilatory function. *A*: the correlation between state 4 respiration and the greatest flow-mediated vasodilatory response (45 µ,l/min) in 9 young (3 males and 6 females) and 15 old (12 males and 3 females) subjects. *B*: the correlation between state 4 respiration and the greatest ACh-dependent vasodilation (10^{-3}) in 8 young (3 males and 5 females) and 13 old (10 males and 3 females) subjects. *C*: the correlation between state 4 respiration and the greatest sodium nitroprusside (SNP)-induced vasodilation (10^{-3}) in 5 young (2 males and 3 females) and 8 old (6 males and 2 females) subjects.

Relationship between RCR and vasodilatory function. When RCR state 3 (CI + II)/state 4 respiration was calculated, there was evidence of a strong and significant positive correlation between this index of net oxidative coupling rate and endothelium-dependent vasodilation, as assessed by both flow-mediated and ACh-dependent vasodilation ($r = 0.85$ and 0.86, $P < 0.05$, respectively; Fig. 5, *A* and *B*). In contrast, there was no evidence of a relationship between RCR and the endothelium-independent SNP-induced vasodilatory response (Fig. 5*C*).

Impact of a mitochondrial-targeted antioxidant on flow- mediated and ACh-dependent vasodilation. To provide mechanistic insight into the findings of the main investigation, an additional interventional investigation was performed. SMFA flow-mediated vasodilation after incubation with the mitochondrial-targeted antioxidant MitoQ was significantly enhanced in the old (control: 29 ± 3; MitoQ: 67 ± 3%) but was not significantly different in the young (control: 66 ± 4; MitoQ: 66 ± 3%; Fig. 6*A*). SMFA ACh-dependent vasodilation after incubation with the mitochondrial-targeted antioxidant MitoQ was significantly enhanced in the old (control: 61 ± 4; MitoQ: 93 ± 3%) but was not significantly different in the young (control: 98 ± 1; MitoQ: 99 ± 2%; Fig. 6*B*).

Pooled data to augment statistical power of the correlation analyses between oxidative phosphorylating respiration and both flow-mediated vasodilation and age. Because of the relative scarcity of human SMFAs across the complete adult age spectrum, additional data from our group's previous studies using the same, but less comprehensive, assessments were pooled with data from the main investigation, augmenting statistical power of the correlation analyses between SMFA mitochondrial respiratory function with both vascular function and age ($n = 46$). Interestingly, as shown in Fig. 7, this very-well-powered analysis revealed a strong overall relation- ship between these three variables ($r = 0.8$, $P < 0.05$; Fig. 7), with state 3 (CI + II) respiration and flow-mediated vasodilation both falling with advancing age ($r = -0.71$ and -0.85, respectively, $P < 0.05$), as well as flow-mediated vasodilation and state 3 (CI + II) respiration being positively correlated with each other ($r = 0.78$, $P < 0.05$; Fig. 7), yielding the following interaction model: flow-mediated dilation(%) 121.8 - (1.6Xage) + (0.06XageXstate 3). Interestingly, this analysis also revealed an interaction between flow-mediated vasodilation and state 3 (CI + II) respiration.

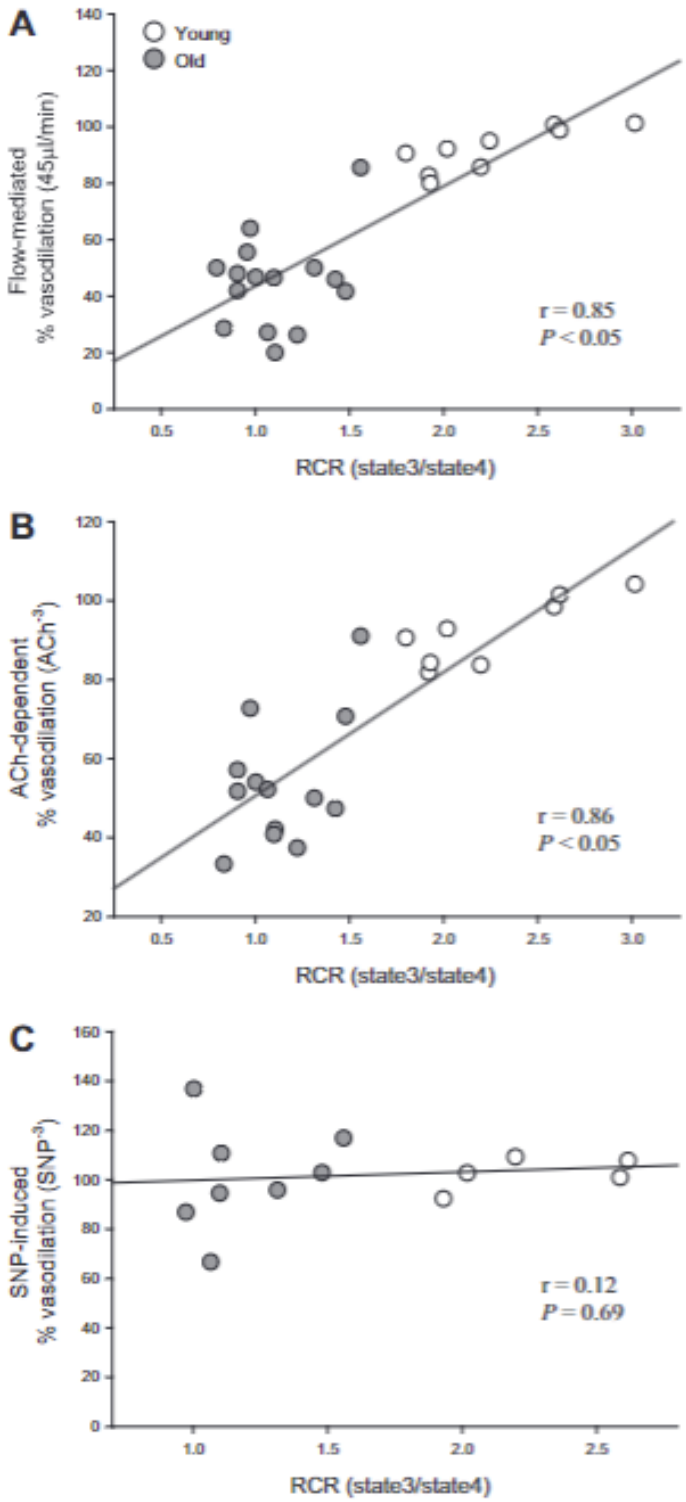

Fig. 5. The relationship between respiratory control ratio (RCR) and vasodilatory function. *A*: the correlation between RCR and the greatest flow-mediated vasodilatory response (45 µ,l/min) in 9 young (3 males and 6 females) and 15 old (12 males and 3 females) subjects. *B*: the correlation between RCR and the greatest ACh-dependent vasodilation (10^{-3}) in 8 young (3 males and 5 females) and 13 old (10 males and 3 females) subjects. *C*: correlation between RCR respiration and the greatest sodium nitroprusside (SNP)-induced vasodilation (10^{-3}) in 5 young (2 males and 3 females) and 8 old (6 males and 2 females) subjects.

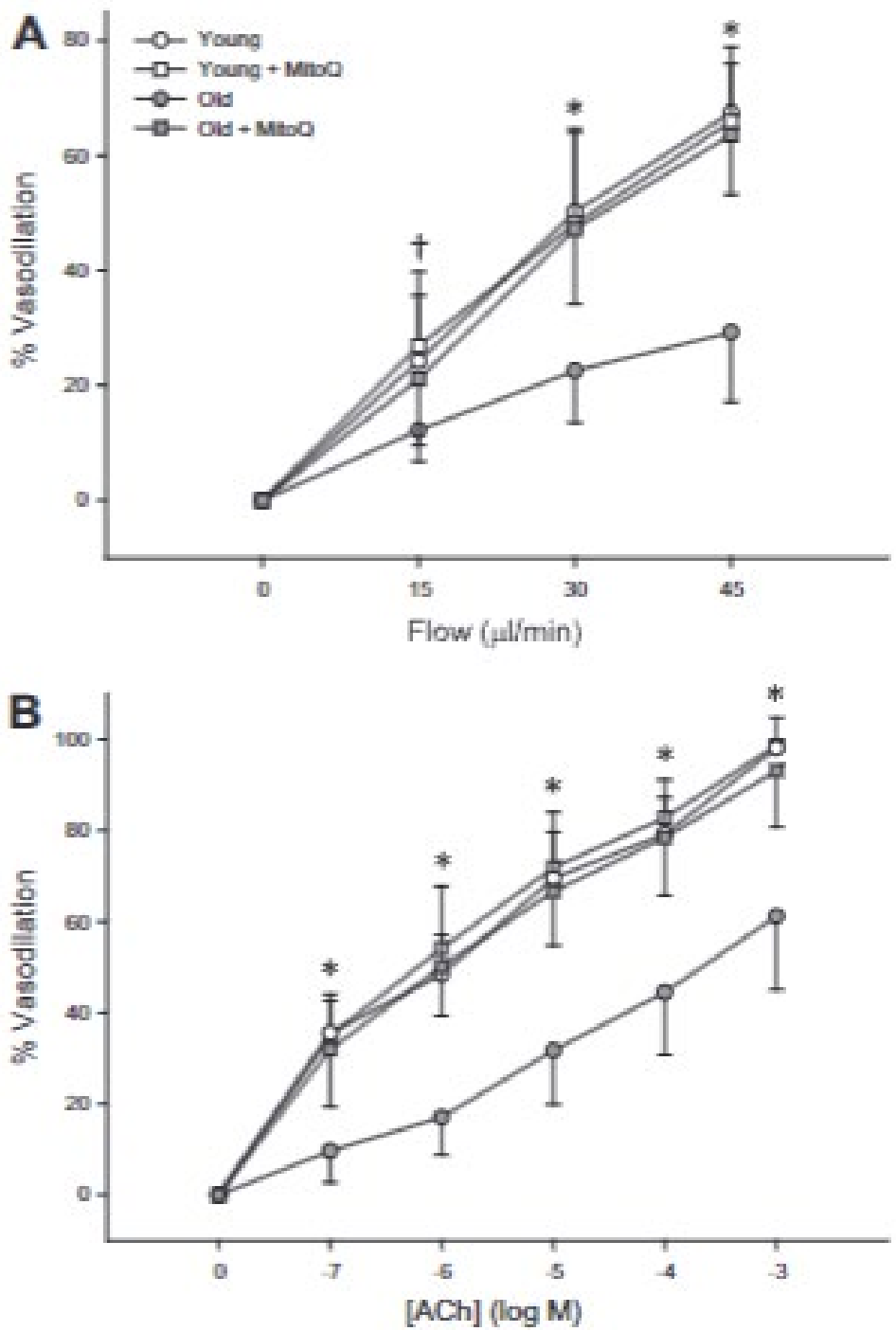

Fig. 6. The vasodilatory capacity of skeletal muscle feed arteries from young and old with and without a mitochondrial-targeted antioxidant (MitoQ). *A*: the vasodilation curves of skeletal muscle feed arteries from young and old subjects with and without MitoQ, induced by different flow rates (15, 30, and 45 µ,l/min), in 9 young (5 males and 4 females) and 13 old (8 males and 5 females) subjects. Brackets denote concentration. Data are expressed as means ± SE. Two-way repeated ANOVA was used. *Significant difference, old versus young, young + MitoQ, and old + MitoQ, $P < 0.05$. †Significant difference, old versus young and young + MitoQ, $P < 0.05$ *B*: the vasodilation curves of skeletal muscle feed arteries from young subjects and old subjects with and without MitoQ induced by different acetylcholine (ACh) concentration. Data are expressed as means ± SE. Two-way repeated ANOVA was used. *Significant difference, old versus young, young + MitoQ, and old + MitoQ, $P < 0.05$.

DISCUSSION

The goal of this study was, for the first time, to examine the link between vascular and mitochondrial respiratory function in the vasculature with advancing age. The main finding of this study was that diminished age-related vasodilatory function is strongly linked to falling mitochondrial respiratory function. Specifically, endothelium-dependent vasodilation, both flow- and ACh-induced, and mitochondrial oxidative phosphorylation, measured as state 3 (CI + II) respiration, were attenuated in old SMFAs compared with the young. Indeed, and of importance, endothelium-dependent, but not endothelium-in- dependent, vasodilatory function was positively correlated with vascular oxidative phosphorylating capacity. Additionally, nonphosphorylating proton leak, an index of free radical production, measured as state 4 respiration was not changed but was negatively correlated with ACh-dependent vasodilation. Furthermore, RCR, an indicator of oxidative coupling efficiency, calculated as state 3 (CI + II)/state 4 respiration, was greatly attenuated in old SMFAs compared with the young and strongly positively correlated with endothelium-dependent vasodilation. Finally, a mitochondrial-targeted antioxidant significantly improved endothelium-dependent vasodilation in old SMFAs compared with the young. Therefore, the age-related decline in vasodilatory function in humans is related to a concomitant attenuation in mitochondrial respiratory function, and this is likely a consequence of augmented free radical production.

Age-related alterations in vasodilatory and vascular mitochondrial respiratory function. It has previously been documented that human SMFAs have the potential to regulate skeletal muscle blood flow, and SMFA vascular function is negatively affected by advancing age (16, 31, 32). The current study confirms this previous work, again documenting that old SMFAs exhibit blunted endothelium-dependent vasodilation compared with the young, as assessed by the flow- and ACh- induced vasodilatory response (Fig. 1, *A* and *B*). This blunted vasodilatory function with advancing age is in line with the primary role of endothelium-derived nitric oxide (NO) in vascular function, especially since the endothelium-independent SNP response was not altered by aging (Fig. 1*C*). There- fore, the observed SMFA dysfunction is mediated by the endothelium, and any age-related alterations in the contractile properties of the vascular smooth

muscle play a minimal role in the documented vasodilatory dysfunction associated with the aging process.

In terms of mitochondrial function, it is well documented that, with advancing age, mitochondrial respiratory function declines in tissue with, relatively, high metabolic demand, such as cardiac and skeletal muscle (4, 8, 34, 40). However, until recently (29), little was known about the impact of age on the respiratory function of the less energetic vascular smooth muscle. This prior work, by our group (29), revealed that, with advancing age, vascular mitochondrial respiratory function declines, and, per mitochondrion, there appears to be a greater likelihood of mitochondrial-derived free radicals. Therefore, the propensity for declining vascular mitochondrial respiratory function in the current study is in agreement with other muscle types. Herein, again, it is documented that, despite state 4 respiration, an index of mitochondrial-derived free radical production, not being significantly different, vascular mitochondrial oxidative phosphorylating respiratory capacity is significantly attenuated in the old compared with the young (Fig. 2). Therefore, this suggests that, despite less vascular mitochondrial mass in the vascular smooth muscle, compared with cardiac and skeletal muscle, due to a very different physiological role (30), the vasculature still exhibits a similar age-related attenuation in mitochondrial respiratory function, as do other muscle types.

Vascular oxidative phosphorylating respiration and age- related SMFA dysfunction. In the vasculature, both mitochondrial function and morphology appear to be altered with advancing age, occurring concomitantly with a fall in mitochondrial content (29, 41). In addition, previous studies have also suggested that impaired oxidative phosphorylation in vascular smooth muscle cells exacerbates vascular inflammation, subsequently leading to the progression of atherosclerosis, which is frequently a characteristic of the aged vasculature (23, 48). In agreement with these prior observations, the current study revealed that the attenuated oxidative phosphorylating capacity, evident with advancing age, is significantly correlated with both flow- and ACh-induced vasodilation (Fig. 3, *A* and *B*). However, it must be acknowledged that these significant relationships were not evident within just the young or old group. Interestingly, this age-related mitochondrial dysfunction was not related at all to the SNP response (Fig. 3*C*), unlike the flow and ACh responses, implying that the

attenuated capacity for oxidative phosphorylating respiration is likely playing a role in the endothelium-mediated function of SMFAs rather than smooth muscle function, per se. Indeed, previous studies have suggested a possible functional link between mitochondrial biogenesis and endothelial NO synthase expression as well as NO bioavailability (1, 28), both of which are associated with the endothelium and are significantly attenuated with advancing age. Thus, in combination, these studies support the premise that age-related endothelium-dependent vasodilatory dysfunction is strongly associated with mitochondrial oxidative phosphorylating respiration in vascular smooth muscle.

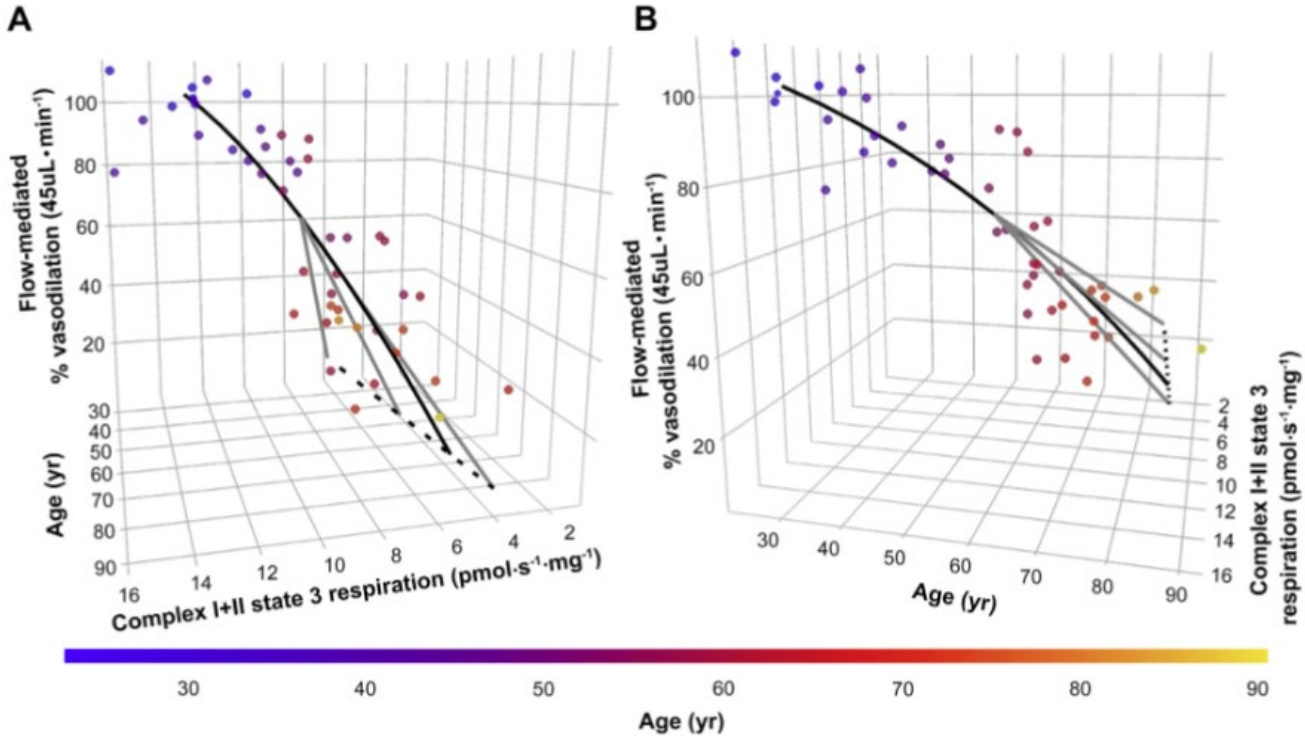

Fig. 7. Pooled data to facilitate an augmented three-dimensional correlation analysis of maximal oxidative phosphorylating respiration and both flow-mediated vasodilation and age, with two view angles (*A* and *B*). There was a strong overall relationship between the three variables ($r = 0.8$, $P < 0.05$), with mitochondrial state 3 (complex I + II) respiration and flow-mediated vasodilation both falling with advancing age ($r = -0.71$ and -0.85, respectively, $P < 0.05$), whereas flow-mediated vasodilation and mitochondrial state 3 (complex I + II) respiration were positively correlated with each other ($r = 0.78$, $P < 0.05$). Additionally, there was a significant ($P < 0.05$) interaction between flow-mediated vasodilation and mitochondrial state 3 (complex I + II) respiration. The gray lines indicate the interaction between aging and state 3 (complex I + II) respiration in the prediction of flow-mediated vasodilation, documenting the finding that the attenuation of both state 3 (complex I + II) respiration and flow-mediated vasodilation can be mitigated in the face of advancing age. This relationship is further summarized by the broken black line that indicates the age by mitochondrial function interaction within the predicted model at age 85. The gray scaling of the symbols represents age from young (light gray) to old (black), and the legend is the gray scale bar to the right of the graph. Multiple regression analysis was used, $n = 46$ total (29 males and 17 females).

Until the current study, the capacity for O_2 flux through mitochondria in the vasculature and the impact on the functional properties of the vessels had yet to be comprehensively studied. Indeed, the current study is the first to examine the link between mitochondrial respiratory capacity and vasodilatory function (Fig. 3). These findings reveal that the age-related decline in endothelium-dependent vasodilatory function in hu- mans is related to a concomitant attenuation in mitochondrial respiratory capacity. However, this is not, actually, so surprising when the powerful influence of metabolic state on cellular homeostasis is considered. For example, increased ATP syn- thesis contributes to the deactivation of AMP-activated protein kinase, a cellular energy sensor monitoring the ADP-to-ATP ratio, leading to a less-suppressing cell cycle in young cells compared with their senescent counterparts (44). In addition, augmented mitochondrial respiration with elevated ATP pro- duction can inhibit cell death by attenuating the release of substances, such as cytochrome c and apoptosis-inducing factors (21), better maintaining cellular homeostasis. However, in the current scenario, as will be addressed in the vascular nonphosphorylating respiration section, free radicals likely play a role.

To improve statistical power and to confirm the strength of the relationship between vasodilatory and vascular mitochondrial respiratory function, as well as age itself, we combined the current oxidative phosphorylating and vascular function data with additional data collected previously, using the same methods. This augmented the statistical power of the correlation analyses between SMFA mitochondrial oxidative capacity and both vascular function and age (n = 46). Interestingly, as shown in Fig. 7, this very-well-powered analysis revealed a strong overall relationship between these three variables, with state 3 (CI + II) respiration and flow-mediated vasodilation both falling with advancing age, as well as flow mediated vasodilation and state 3 (CI + II) respiration being positively correlated with each other. Interestingly, there was an interaction between flow-mediated vasodilation and state 3 (CI + II) respiration, which suggests that, in the older subjects, there is evidence to indicate that those subjects with good mitochondrial respiratory function would exhibit better vascular function and vice versa. These additional data analyses add significant credence to the conclusion that the age-related decline in vasodilatory function in humans is related to a

concomitant attenuation in mitochondrial respiratory function.

Vascular nonphosphorylating respiration, oxidative coupling efficiency, and age-related SMFA dysfunction. Free radicals are produced as a product of ATP synthesis and have been implicated as a primary cause of age-related vascular dysfunction (41, 42). In this study, the difference in the level of nonphosphorylating proton leak (state 4 respiration), an index of free radical production, between young and old subjects did not achieve statistical significance (Fig. 2). However, interestingly, the correlation analysis revealed that nonphosphorylating state 4 respiration was negatively related to ACh-dependent vasodilation but was clearly unrelated to the SNP response (Fig. 4, *A*–*C*). This implies that mitochondrial-derived free radicals, potentially, have a negative impact on vascular endothelial function with advancing age.

There have been several in vivo and in vitro studies that have revealed that targeting mitochondrial-derived free radical pro- duction can successfully ameliorate age-related vasodilatory dysfunction in both humans and animals (11, 32, 36, 41, 46, 47). In general, although achieving the appropriate level of mitochondrial free radical production is well documented to play a critical role in molecular signaling by determining redox balance (8, 9, 11), free radicals produced from the mitochondrial respiratory chain, specifically complex I, have been documented to play a pivotal role in vascular dysfunction with advancing age (26, 43). This is supported by the current data in that complex I-driven state 3 respiration capacity was significantly correlated with both flow- and ACh-induced vasodilation capacity while complex II-driven state 3 capacity was not. Recognizing that, due to tissue volume and methodology, the current metabolic assessments are heavily weighted toward the vascular smooth muscle, these results highlight the potentially important role of vascular smooth muscle mitochondria in the recognized endothelial-dependent dysfunction with advancing age. This may, mechanistically, be explained by the NO released from endothelial cells in the elderly, which enter the smooth muscle to initiate vasodilation, being compromised by augmented oxidative stress in the vascular smooth muscle, resulting in attenuated NO bioavailability and vasodilatory function in this population (22). Furthermore, the current performance of the additional interventional investigation, aimed at providing direct mechanistic insight into the findings of the main investigation, revealed that the impact

of the mitochondrial-targeted antioxidant was most evident in the old (Fig. 6, *A* and *B*). In combination, these findings further support the concept that, with advancing age, vascular mitochondrial free radical production is likely exacerbated and mechanistically linked to age-related vasodilatory dysfunction.

The RCR provides a gauge of how effectively mitochondria consume O_2 to produce ATP in response to a given metabolic demand, taking into account the O_2 consumption from proton leak (5). Of note, in the current study, when each subject's phosphorylating respiration, which correlated well with endothelium-mediated vasodilatory function (Fig. 3, *A* and *B*), was corrected for nonphosphorylating respiration (Fig. 3, *A* and *B*), the resultant variable, RCR, was even more strongly correlated with endothelial function (Fig. 5, *A* and *B*). This finding is in agreement with prior studies, in the more energetically active cardiac and skeletal muscle, that have documented a decline in RCR with aging, ultimately resulting in a loss of normal function (12). Thus, it appears that vascular mitochondrial oxidative coupling efficiency likely plays a role in endothelium-dependent vascular function.

Experimental considerations. As with any investigation, this study was not without a variety of experimental considerations. First, because we used an in vitro approach to study SMFA function, it should be acknowledged that such an approach does not replicate the vastly more complex situation in vivo. Furthermore, although great effort was expended to study a simplified in vitro paradigm, cleaning and rinsing the vessels, it is possible that age-related changes in cells, other than endothelial and smooth muscle cells (e.g., adipocytes and macrophages), may have influenced the results of this study. Second, because human SMFAs are difficult to obtain, and the use of 68 vessels to complete this work, the scope of this study was not as broad as equivalent animal work with, for example, the examination of the impact of only one antioxidant (MitoQ). However, despite limited samples, this study clearly documented a link between vasodilatory and vascular mitochondrial respiratory function with advancing age, which is likely mediated by free radicals. Third, the lack of physical activity records or a physical activity assessment raised questions about the role of changing activity with advancing age that cannot be answered by the current study. Fourth, unintentionally in the main investigation, the young and old have a different ratio of sex, with 67% of the

young group's SFMAs being from women, whereas only 20% of the old group's SFMAs were from women. Although this is an unfortunate difference in the ratio of males to females, statistically, there was no main effect of sex in the current findings. Finally, in terms of antioxidant enzymes, the potential for their differential expression affect- ing subsequent free radical formation cannot be excluded; however, it should be noted, that, typically, older subjects often exhibit elevated antioxidant enzymes, and this would tend to attenuate the actual age-related difference in free radicals.

Perspectives and Significance

The goal of this study was, for the first time, to comprehensively examine the link between vasodilatory and vascular mitochondrial respiratory function with advancing age. It was determined that the age-related decline in vasodilatory function in humans is related to a concomitant attenuation in vascular mitochondrial respiratory function. Furthermore, because a mitochondrial-targeted antioxidant significantly improved endothelium-dependent vasodilation, it is likely that the mechanistic link between vasodilatory and vascular mitochondrial respiratory function with advancing age is free radically mediated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.H.P., O.S.K., and R.S.R. conceived and designed research; S.H.P., O.S.K., S.-Y.P., J.C.W., J.R. Hydren, V.R., R.H.I.A., and J.R. Hyngstrom performed experiments; S.H.P., O.S.K., S.-Y.P., J.C.W., J.R. Hydren, and R.S.R. analyzed data; S.H.P.,

O.S.K., and R.S.R. interpreted results of experiments; S.H.P. and R.S.R. prepared figures; S.H.P. and R.S.R. drafted manuscript; S.H.P., O.S.K., R.H.I.A., J.R. Hyngstrom, and R.S.R. edited and revised manuscript; S.H.P., O.S.K., S.-Y.P., J.C.W., J.R. Hydren, R.H.I.A., J.R. Hyngstrom, and R.S.R. approved final version of manuscript.

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