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Abstract

Aim: This study sought to determine the role of free radicals derived from mitochondria in the vasculature in the recognized age-related endothelial dysfunction of human skeletal muscle feed arteries (SMFAs).

Methods: A total of 44 SMFAs were studied with and without acute exposure to the mitochondria-targeted antioxidant MitoQ and nitric oxide synthase (NOS) blockade. The relative abundance of proteins from the electron transport chain, phosphorylated (p-) to endothelial (e) NOS ratio, manganese superoxide dismutase (MnSOD) and the mitochondria-derived superoxide (O^-) levels were assessed in SMFA. Endothelium-dependent and endothelium-independent SMFA vasodilation was assessed in response to flow-induced shear stress, acetylcholine (ACh) and sodium nitroprusside (SNP).

Results: MitoQ restored endothelium-dependent vasodilation in the old to that of the young when stimulated by both flow (young: 68 ± 5; old: 25 ± 7; old + MitoQ 65 ± 9%) and ACh (young: 97 ± 4; old: 59 ± 10; old + MitoQ: 98 ± 5%), but

did not alter the initially uncompromised, endothelium-independent vasodilation (SNP). Compared to the young, MitoQ in the old diminished the initially elevated mitochondria-derived O^- levels and appeared to attenuate the breakdown of MnSOD. Further- more, MitoQ increased the ratio of p-eNOS to NOS and the restoration of endothelium-dependent vasodilation in the old by MitoQ was ablated by NOS blockade.

Conclusion: This study demonstrated that MitoQ reverses age-related vascular dysfunction by what appears to be an NO-dependent mechanism in human SMFAs. These findings suggest that mitochondria-targeted antioxidants may have utility in terms of counteracting the attenuated blood flow and vascular dysfunction associated with advancing age.

With advancing age, blood flow to skeletal muscle is often diminished,[1,2] which, at least in part, is likely a consequence of attenuated endothelial function in the skeletal muscle resistance vasculature.[3–5] However, the specific mechanisms responsible for the age-related attenuation of skeletal muscle blood flow are currently not well understood. The study of human SMFAs is highly germane to better understanding the vascular biology of ageing, as it affords the opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood flow, also have regulatory potential.[6] Indeed, our group has recently documented that the vasodilator function of SMFAs obtained from elderly human subjects was markedly attenuated and this functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS protein levels.[7] Although attenuated NO bioavailability with advancing age may depend on multiple factors that regulate NO production and degradation, free radicals, principally O^-,[8–10] likely play an important role by reacting rapidly with NO, thereby decreasing NO bioavailability.[9,11] Currently, the exact source of the free radicals that appear to

attenuate NO bioavailability and subsequent endothelial dysfunction with advancing age remain unclear.

Mitochondria play a critical role in cellular function in both health and disease, but are also an important and major source of free radicals.[12,13] Interestingly, although mitochondrial content is relatively low in vascular endothelial cells and smooth muscle (2–5% of cell volume) compared to physically active skeletal muscle and cardiac myocytes (5–35% of cell volume),[14] previous studies have revealed a strong correlation between mitochondria-derived oxidative stress and endothelial dysfunction.[8,12,15] Interestingly, our group recently documented that exercise training induces an increase in vascular mitochondrial respiratory capacity, evidence of improved redox balance, and elevated basal NO bioavailability.[16] These data suggest that age- and disease-related alterations in arterial function may be directly affected by the function, and subsequent free radical production, of mitochondria in the vasculature. Therefore, strategies to constrain mitochondria-derived free radical levels to within typical physiological levels may prove useful in attenuating the development of endothelial dysfunction with age.

The first line of defence against free radicals is both endogenous and exogenous antioxidants. However, to date, antioxidant supplementation (e.g. vitamin C) has not proven effective at specifically decreasing mitochondria-derived free radical levels.[17–19] Of note, as mitochondria are negatively charged, the incorporation of a lipophilic cation, such as triphenylphosphonium (TPP), to a potent antioxidant, such as the active ubiquinol moiety of coenzyme Q10, enables the selective and extensive accumulation of the antioxidant within the mitochondria.[20,21] Utilizing this approach, a commercially available mitochondria-targeted antioxidant, MitoQ (MitoQ Limited, Auckland, New Zealand), has been synthesized to yield a thousand-fold greater concentration within the mitochondria than untargeted antioxidants, which distribute throughout the cell.[20,21] The use of MitoQ to specifically treat age-related endothelial function is sup- ported by a recent, elegant and comprehensive study by Gioscia-Ryan *et al.*,[22] who reported that this mitochondria-targeted antioxidant attenuated endothelial dysfunction in

older mice. Nevertheless, age-related vascular mitochondrial free radical levels and endothelial dysfunction in humans have yet to be examined.

Consequently, utilizing the pressure myography technique and incubation with MitoQ, this study sought to determine the role of free radicals derived from vascular mitochondria in the age-related endothelial dysfunction of human SMFAs. We tested the hypothesis that free radicals derived from vascular mitochondria play a critical role in attenuating NO bioavailability and, subsequently, promote endothelial dysfunction in the elderly.

Results

Subject characteristics

From the 44 SMFAs that were harvested, 18 were from young subjects (33 ± 2 years) and 26 were from old subjects (72 ± 5 years). The subject characteristics, retrospectively obtained from pre-operative examination of medical records, are presented in Table 1. Note that users of cancer-related medications were excluded from the study. Also, it should be noted that all blood biochemical analysis and complete blood count results (Table 1) were within normal ranges, suggesting that the subjects who participated in this study were relatively healthy.

Vessel characteristics

Skeletal muscle feed arteries were harvested from either the inguinal ($n = $ 23) or axial ($n = 21$) regions from either males ($n = 25$) or females ($n = 19$). In agreement with our previous observations, vessel function was not different as a consequence of anatomic origin or sex. Immunoblotting, to assess the relative abundance of proteins in the electron transport chain, revealed that the majority of the mitochondrial respiratory complexes, with the exception of complex V, were significantly attenuated in the SMFAs of the old compared to the young (Fig. 1). MitoQ did not alter this attenuation of the mitochondrial respiratory complexes in the old (Fig. 1). Basal, unpressurized, outer diameter of the SMFAs was not statistically different in the young, old, and old with MitoQ (young: 510 ± 12 µm; old: 514 ± 15 µm; old+MitoQ:515 ± 10 µm). Additionally, maximal outer diameter

of the SMFAs, achieved by incubation in Ca^{2+}-free physiological salt solution (PSS), was not statistically different in the young, old and old with MitoQ (young: 758 ± 19 µm; old: 752 ± 14 µm; old+Mi-toQ: 750 ± 15 µm).

Table 1 Subject characteristics

	Young (*n*=18)	Old (*n*=26)
Age (year)	32 ± 6	75 ± 7*
Sex (male/female, *n*)	10/8	15/11
Height (cm)	175 ± 15	165 ± 12
Body mass (kg)	74 ± 13	81 ± 10
BMI (kg m^{-2})	21 ± 7	27 ± 7
Systolic blood pressure (mmHg)	116 ± 7	126 ± 9
Diastolic blood pressure (mmHg)	78 ± 5	81 ± 9
Glucose (mg dL^{-1})	110.8 ± 9.2	108 ± 5.2
Blood urea nitrogen (mg dL^{-1})	17.4 ± 5.0	16.8 ± 6.4
Creatinine (mg dL^{-1})	0.9 ± 0.7	1 ± 0.9
Albumin (g dL^{-1})	4.2 ± 0.6	4.2 ± 0.7
Lactate dehydrogenase (U L^{-1})	505.4 ± 40.1	503 ± 47.3
Haemoglobin (g dL^{-1})	26.5 ± 1.2	14.3 ± 1.5
White blood cells (thousands per microlitre, K µL^{-1})	4.9 ± 2.1	7.7 ± 1.4
Red blood cells (millions per microlitre, M µL^{-1})	5.2 ± 1.3	4.8 ± 1.5
Platelets (K µL^{-1})	255.9 ± 21.1	240 ± 27.2
Haematocrit (%)	41.4 ± 3.1	40 ± 5
Lymphocytes (%)	34.3 ± 3.3	33 ± 8.5
Monocytes (%)	8.6 ± 1.6	8.1 ± 2.5
Medications (Users/n)		
Diuretics	0/18	2/26
Angiotensin-converting enzyme inhibitors	0/18	2/26
Diabetic drugs	0/18	3/26
Statins	0/18	2/26

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

The vasodilator response to flow, ACh and SNP and the impact of MitoQ in the old

The PE-induced pre-constriction of the SMFAs prior to the flow stimulus was similar between groups (young: 69 ± 4%; old: 67 ± 5%; old + MitoQ: 68 ± 5, *P* > 0.05). The greatest vasodilation in response to the intraluminal flow of 45 ± 3 µl min^{-1} was significantly attenuated in the old compared to the young (young: 68 ± 5; old: 25 ± 7%, *P* < 0.05; Fig. 2a). However, the vasodilator response to flow in the old was restored to that of the young by MitoQ (old + MitoQ: 65 ± 9%; Fig. 2a). This effect of MitoQ in the old was also evident at the lower intraluminal flow rates of 15 ± 2 and 30 ± 4 µl min^{-1} (Fig. 2a).

The PE-induced pre-constriction of the SMFAs prior to the ACh and SNP dose–response curves was similar between groups (young: $69 \pm 4\%$; old control: $68 \pm 5\%$; old + MitoQ $69 \pm 5\%$, $P > 0.05$). The greatest vasodilation in response to the highest dose of ACh (10^{-3} M) was significantly attenuated in the old compared to the young ACh (young: $97 \pm 4\%$; old: $59 \pm 10\%$, $P < 0.05$) (Fig. 2b). However, the vasodilator response to ACh in the old was restored to that of the young by MitoQ (old + MitoQ: $98 \pm 5\%$; Fig. 2b). This effect of MitoQ in the old was clearly evident across the whole ACh dose–response curve (Fig. 2b). In contrast, endothelial-independent vasodilator function, the vasodilator response to the highest dose of SNP (10^{-4} M; young: $97 \pm 4\%$; old: $100 \pm 11\%$; old + MitoQ: $98 \pm 4\%$, $P > 0.05$) and across the whole dose–response curve, was similar among the young, old and old with MitoQ (Fig. 2c).
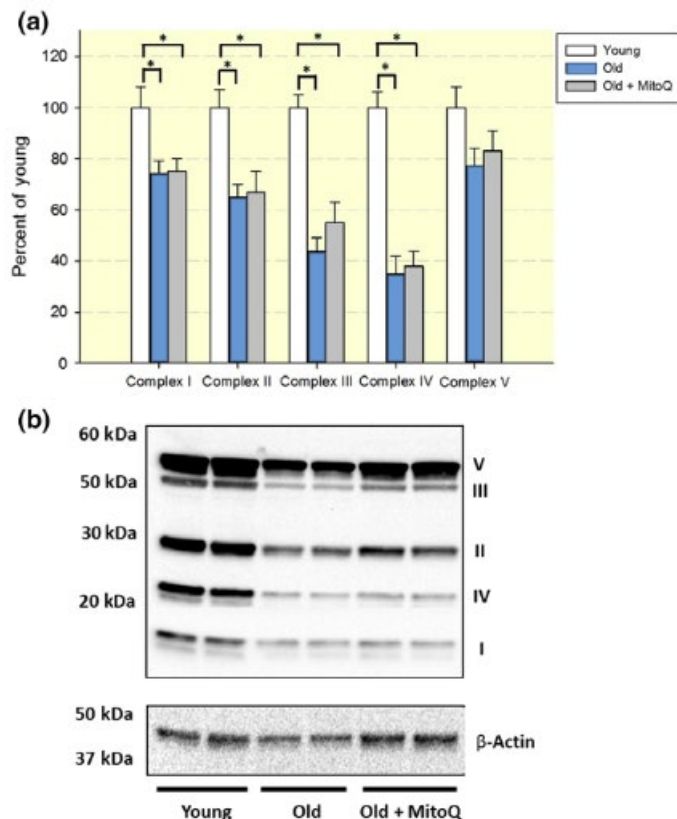

Figure 1 The relative abundance of skeletal muscle feed artery proteins from the electron transport chain (ETC.) of young subjects and old subjects with and without MitoQ. The electron transport chain protein expression was normalized by β-actin protein expression. Data are expressed as mean ± SE. $n = 10$ young and 16 old subjects. *Significantly different from young vs. old and young vs. old + MitoQ, $P < 0.05$.
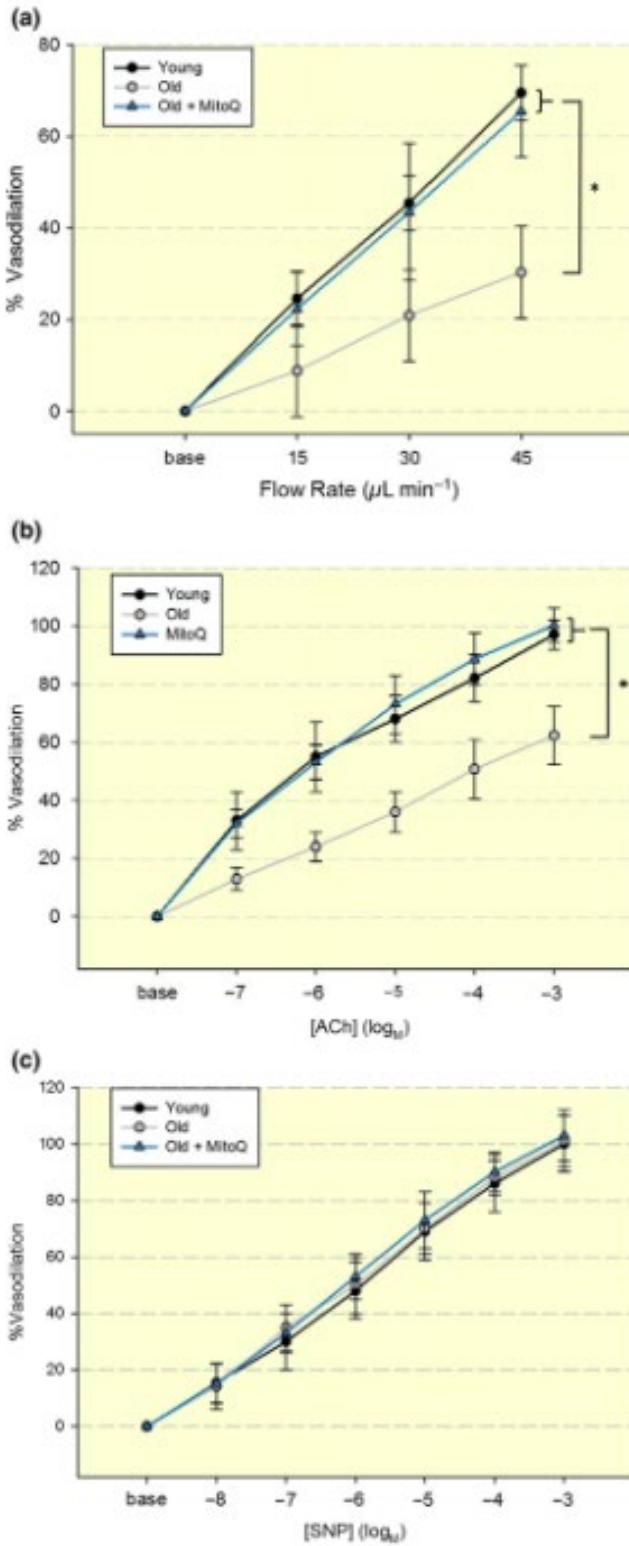
Figure 2 The vasodilator dose–response curves of skeletal muscle feed arteries from young subjects and old subjects with and without MitoQ evoked by flow, acetylcholine (ACh) and sodium nitroprusside (SNP). Data are expressed mean ± SE. $n = 10$ young and 16 old subjects. *Significantly different from young and old + MitoQ vs. old, $P < 0.05$.

Levels of mitochondria-specific O_2^- and MnSOD and the impact of MitoQ in the old

The baseline EPR spectroscopy signal for the mito- Tempo-H adduct in the SMFAs, an index of mitochondria-specific O_2^- levels, was greater in the old compared to the young (young: 1.7 ± 0.2; old: 6 ± 1.8; AUC/mg, $P < 0.05$; Fig. 3a). However, MitoQ significantly lowered SMFA O_2^- levels in the old, such that the old was similar to the young (old + MitoQ: 1.95 ± 0.7; AUC/mg; Fig. 3a). In terms of antioxidant status, immunoblotting revealed that baseline MnSOD protein content was significantly attenuated in the old compared to the young (young: 100 ± 16; old: 35 ± 18; AU $P < 0.05$; Fig. 3b). However, following incubation with MitoQ, MnSOD protein content in the old was greater than in control conditions (old + MitoQ: 59 ± 18 AU) (Fig. 3b), which could be interpreted as significantly attenuating MnSOD breakdown. It is important to note that although the pre-incubation period was only 1 h in length, when assessed for protein content, at the end of these studies, the SMFAs had typically been exposed to MitoQ or control conditions for ≈4 h.
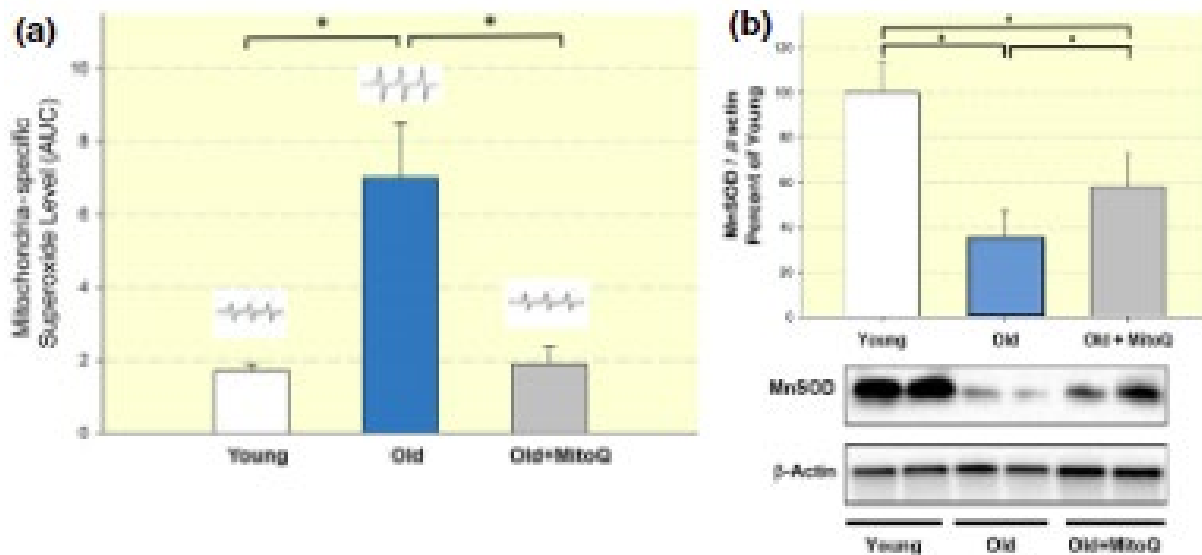

Figure 3 Mitochondria-specific superoxide levels and manganese superoxide dismutase (MnSOD) protein expression in skeletal muscle feed arteries of young subjects and old subjects with and without MitoQ. Superoxide levels were assessed utilizing the mitochondria-specific superoxide spin trap mitoTempo-H and electron paramagnetic resonance (EPR) spectroscopy. The EPR signal was expressed as the area under the curve (AUC) in arbitrary units and representative spectra are inlayed. The MnSOD protein expression was normalized by b-actin protein expression. Data are expressed as mean ± SE. *n* = 8 young and 8 old subjects for EPR and *n* = 8 young and 10 old subjects for immunoblotting. *Significantly different from young and old + MitoQ, $P < 0.05$.

The role of NO bioavailability and the impact of MitoQ in the old

Skeletal muscle feed arteries immunoblotting revealed that eNOS protein content, normalized for beta-actin, was significantly lower in the old compared to the young (young: 100 ± 24; old: 55 ± 20; AU $P < 0.05$) and was unaffected by MitoQ (old + MitoQ: 62 ± 17 AU) (Fig. 4). Furthermore, eNOS phosphorylation, measured as the p-eNOS/eNOS ratio on the western blots, was significantly lower in the old compared to the young (young: 1 00 ± 18; old: 38 ± 17 AU, $P < 0.05$). However, MitoQ enhanced eNOS phosphorylation in the old (old + MitoQ: 78 ± 15 AU; Fig. 4). SMFA vasodilation, in response to both flow and increasing doses of ACh, again revealed attenuated endothelial-dependent vasodilation in the old, which could be restored acutely by MitoQ (Fig. 5a,b). However, the impact of the MitoQ was negated by NOS blockade (Fig. 5a,b). Furthermore, in the presence of L-NMMA, the vasodilator response to both flow and ACh with and without MitoQ was attenuated to a level that was significantly lower than the initial dose response in the old (Fig. 5a,b).
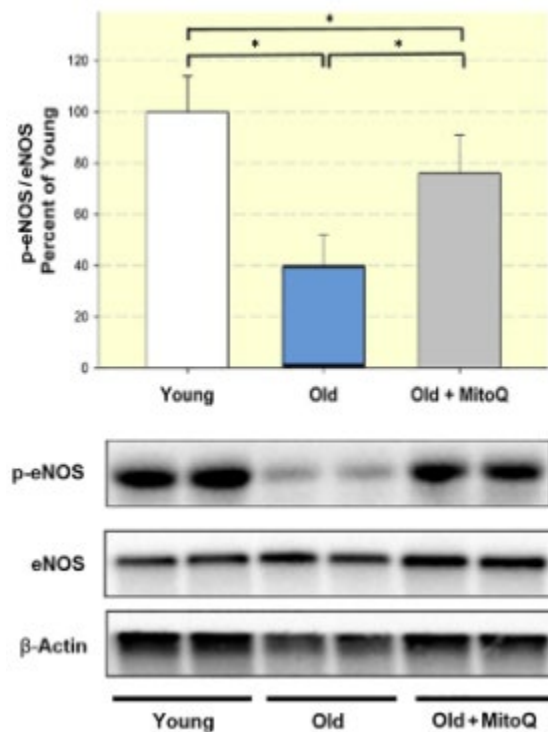

Figure 4 The relative abundance of proteins for endothelial NOS (eNOS) and phosphorylated (p-) eNOS at Ser1177 from skeletal muscle feed arteries of young subjects and old sub- jects with and without MitoQ. Data are expressed as mean ± SE. n = 8 young and 10 old subjects. *Significantly different from young and old + MitoQ vs. old, and young vs. old + MitoQ, $P < 0.05$.

Discussion

This study sought to determine the role of free radicals derived from mitochondria in the vasculature in the age-related endothelial dysfunction documented in human SMFAs. The main hypothesis tested by this investigation was that free radicals derived from ageing vascular mitochondria play a critical role in attenuating NO bioavailability and, subsequently, promote endothelial dysfunction in the elderly. The current findings strongly support this postulate and, of importance, translate previous findings in an animal model to humans. Specifically, despite the observation that the electron transport chain proteins were lower in the old, and this was not altered by MitoQ, this mitochondria-targeted antioxidant acutely restored SMFA endothelium-dependent vasodilation, in response to both flow and ACh, to that of the young. Additionally, MitoQ attenuated mitochondria-derived O_2^- levels, which could be interpreted as a decrease in MnSOD protein breakdown. Furthermore, in the old, the restoration of SMFA endothelium-dependent vasodilation by MitoQ was ablated by NOS blockade, and eNOS phosphorylation was greater in the presence of MitoQ. Thus, augmented mitochondrial free radical levels in the SMFAs of the elderly appear to play a critical role in attenuating NO bioavailability and, subsequently, promoting endothelial dysfunction with advancing age.

Vascular ageing, SMFAs, free radicals and NO bioavailability

In terms of the vascular biology of ageing, the study of human SMFAs is pertinent, as it affords the opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood flow, also have regulatory potential.[6] In fact, our group recently documented that the endothelial function of SMFAs attained from the elderly was markedly attenuated and this functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS protein level, emphasizing the likely role of attenuated NO bioavailability.[7] Here, the findings of this previous work were confirmed with further evidence that ageing similarly attenuates both flow- and ACh-mediated vasodilation in SMFAs (Fig. 2a,b), each indicators of endothelium-dependent vasodilation. The current findings further
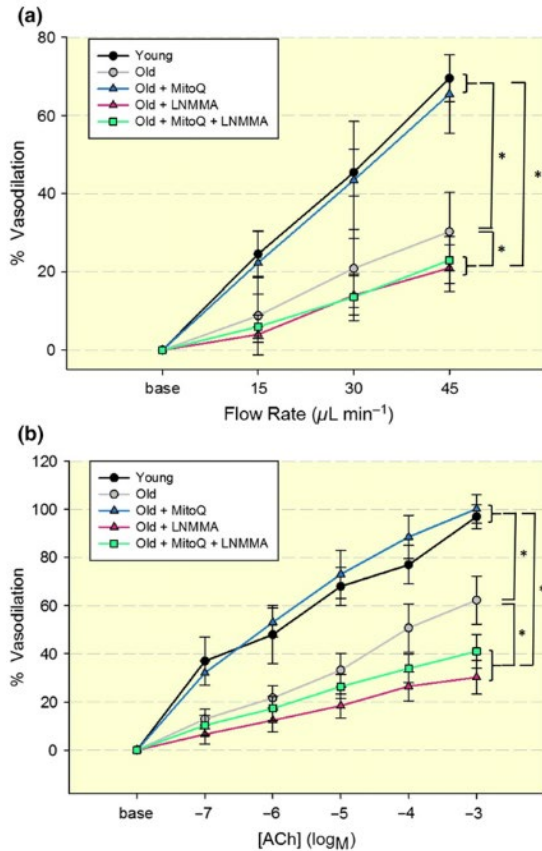
Figure 5 The vasodilator dose–response curves of skeletal muscle feed arteries from young subjects and old subjects both with and without MitoQ and with and without nitric oxide synthase blockade (L-NMMA) evoked by both flow and acetylcholine (ACh). Data are expressed as mean ± SE. n = 8 young and 10 old subjects. Panel (a): *significantly different from young and old + MitoQ vs. old, and old vs. old + LNMMA and old + MitoQ + LNMMA only at 30 and 45 µl min^{-1}, $P <$ 0.05; Panel (b): *significantly different from young and old + MitoQ vs. old, and old vs. old + LNMMA and old + MitoQ + LNMMA only at 10^{-5}, 10^{-4} and 10^{-3} ACh concentration, $P <$ 0.05.

suggest that this limited vasodilator capacity with advancing age is, at least in part, due to attenuated NO bioavailability, as again evidenced by a decrease in the ratio of p-eNOS to total eNOS protein expression in the SMFAs from the old[7] (Fig. 4). Attenuated NO bioavailability with advancing age depends on multiple factors that regulate NO production and degradation, with a key role being played by free radicals. For example, O_2^- decreases NO bioavailability[8–10] by rapidly reacting with NO to form peroxynitrite (ONOO$^-$), but then, in turn, ONOO$^-$ may oxidize the essential cofactor for eNOS, tetrahydrobiopterin, resulting in O_2^- production, rather than NO, by eNOS.[9,11] This redox imbalance likely plays an important role in the age-related fall in NO bioavailability, supported in this study by the greater mitochondria-derived O_2^- levels and appeared to reciprocally

attenuate MnSOD protein breakdown in the old SMFAs (Fig. 3a,b). Indeed, there is accumulating evidence that increased free radical production leads to endothelial dysfunction with advancing age both in animals and in humans and that the resultant oxidative stress promotes vascular disease.[19,23,24]

MitoQ, age-related vascular dysfunction and NO bioavailability

The acute 1-h incubation of the SMFAs from the old with MitoQ effectively reversed the age-related vascular dysfunction (Fig. 2a,b). Several lines of evidence from this study suggest that this restoration of vascular function in the old SMFAs was NO mediated. First, MitoQ greatly attenuated mitochondrial O_2^- levels to more closely resemble that of the young (Fig. 3a), a change that would likely result in an increase in NO bioavailability. Again, it is interesting to note that this fall in O_2^- levels appeared to be concomitant with the attenuated MnSOD breakdown in the old with MitoQ incubation (Fig. 3b). This makes intuitive sense and suggests a MitoQ-induced 'sparing' of MnSOD, an endogenous antioxidant that targets O- and is found predominantly within the mitochondria. Such a preservation of MnSOD protein content in the old with MitoQ is supported by similar prior work from our group,[19] which documented an increase in plasma SOD activity 60 min following the administration of an antioxidant cocktail (vitamins C, E and alpha lipoic acid) in both young and old sub- jects. Second, MitoQ significantly increased the attenuated ratio of p-eNOS to total eNOS protein expression in the SMFAs from the old (Fig. 4), indicative of rescuing the activity of this NO-producing pathway. Third, the reversal of the age-related vascular dysfunction achieved by MitoQ during both the flow and ACh dose–response curves was ablated by NOS blockade, confirming a role for NOS in the MitoQ-induced response. Furthermore, the flow and ACh responses with and without MitoQ, in combination with NOS blockade, were significantly attenuated compared to the flow and ACh assessments in the old SMFAs. Overall, this indicates that NO still plays a role in the response of the old vessels, but, more importantly, that MitoQ was ineffectual when NOS was blocked, implying an NO-mediated mechanism of action (Fig. 2a,b). Although performed in stroke- prone

hypertensive rats, the conclusion by Graham *et al.*[25] that MitoQ supplementation, initiated prior to the establishment of cardiovascular disease (CVD) in young animals, prevented the development of endothelial dysfunction by maintaining NO bioavailability is in agreement with the premise of the current findings.

Vascular ageing, SMFAs, blood flow and oxygen transport

It is widely accepted that ageing is commonly associated with impaired blood flow, and subsequently oxy- gen delivery, to skeletal muscle during dynamic exercise and that this is likely caused by a combination of compromised cardiac output[26,27] and attenuated peripheral vascular conductance with age.[23,27] In terms of the skeletal muscle vasculature, in rodent studies, the rate of endothelium-dependent vasodilation in the skeletal muscle arterioles, which are downstream from the SMFAs, and microcirculatory blood flow were attenuated in old compared to young animals,[28,29] subsequently impairing oxygen delivery to the contracting muscles. In humans, our group recently provided evidence supporting the contention that human SMFAs, the inlets to the muscle bed upstream of the arterioles, regulate vascular resistance, and therefore skeletal muscle perfusion, in response to shear stress and pharmacological vasodilators.[7,26] Furthermore, our group has also demonstrated that SMFAs from older humans exhibit an attenuated magnitude of endothelium- dependent vasodilation and delayed vasodilation kinetics in response to shear stress and ACh.[7] In agreement with these prior results, the current findings confirm that the endothelium-dependent vasodilator capacity of SMFAs, assessed by flow-induced shear stress and the response to ACh, is clearly attenuated with advancing age (Fig. 2a,b). This attenuated SMFA vasodilation with ageing is likely one of the mechanisms responsible for the age-related decline in blood flow and oxygen transport to active skeletal muscle during physical activity in the elderly. In the light of the current positive findings with MitoQ and the positive impact on age-related vascular function, additional studies examining the effect of mitochondria-targeted antioxidants on skeletal muscle blood flow during exercise in the elderly are warranted.

Mitochondrial health, vascular ageing and MitoQ

As the major energy producers for most physiological processes, well-functioning, healthy mitochondria are essential for both systemic and cellular homeostasis. However, in addition to a central role in energy pro- duction, mitochondria seem to be important in terms of molecular signalling and cellular secretion in the vasculature and this is mediated, at least to some extent, by free radicals.[14,15] Indeed, free radicals, produced at numerous sites within the mitochondria, including complexes I, II and III of the electron trans- port chain, play a critical role in these processes. For example, it has been documented that mitochondria located in the endothelial cytoskeleton of arterioles, in the human myocardium, produce free radicals in response to shear stress-induced cell deformation, which are critical for flow-mediated dilation.[30] Conversely, several recent studies have also revealed that mitochondria-derived free radicals in the vasculature play a critical role in peripheral vascular dysfunction with advancing age.[22,31,32] Interestingly, and along these lines, both hyperglycaemia and elevated triglycerides, recognized as inducers of endothelial dysfunction and atherosclerosis, increase mitochondria-derived free radicals and alter mitochondrial dynamics in vascular endothelial cells. This vascular dysfunction can be reversed by normalizing the blood glucose and lipid load, removing the mitochondrial stimulus.[33] Furthermore, and perhaps somewhat ironically, in terms of mitochondrial health, mitochondria-derived free radicals lower the abundance of MnSOD, which resides in the mitochondrial matrix, and negatively impacts mitochondrial biogenesis and mitochondrial content.[22]

The initial age-related findings from this study sup- port the link between attenuated vascular function with advancing age (Fig. 2a,b) and compromised vascular mitochondrial health, as evidenced by the greater O_2^- levels (Fig. 3a), lower levels of MnSOD (Fig. 3b) and the attenuation of the electron transport chain complexes (Fig. 1) in the SMFAs from the old. Interestingly, in addition to restoring endothelial function in the SMFAs from the old (Fig. 2a,b), the acute 1-h incubation with MitoQ both decreased mitochondrial O_2^- levels (Fig. 3a) and preserved mitochondrial antioxidant capacity (MnSOD) (Fig. 3b). However,

MitoQ did not impact the relative abundance of electron transport chain complex proteins (Fig. 1). This is of particular relevance in the light of recent studies that have suggested ageing is associated with attenuated mitochondrial respiratory complexes[34] and that elevated mitochondria-derived free radical production damages the mitochondrial DNA that encodes the electron transport chain complexes.[35,36] This damage, predominantly at complex I, appears to directly affect electron transport and disrupts the whole mitochondrial respiratory cycle.[35,36] In the current study, although perhaps not surprising, due to the relatively short time course of the MitoQ exposure, the lack of effect on the significantly attenuated electron transport chain complex protein expression is an important observation. Specifically, this documents that the positive impact of MitoQ on vessel function and mitochondrial free radical levels is not dependent upon more long-term changes in the relative abundance of the mitochondrial complexes.

Experimental considerations

Although not the main focus of the current study, it is interesting to note that MnSOD protein content in the old SMFAs was attenuated in control conditions com- pared to the young and was more abundant than in these control conditions when incubated in MitoQ. We interpreted these findings as evidence of a greater MnSOD breakdown in the old due to greater oxidative stress and sparing of MnSOD by the antioxidant action of MitoQ. However, without an assessment of MnSOD gene expression, there is a degree of uncertainty in this interpretation. Indeed, prior studies have documented that nuclear factor (erythroid-derived 2)- like 2 (Nrf2), a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins including MnSOD, is diminished or functionally com- promised with advancing age.[37–39] Hence, it is possible that MnSOD gene expression, potentially mediated by Nrf2, was responsible for the documented fluctuations in MnSOD protein content. Unfortunately, due to the scarcity of human SMFAs and the use of all current samples for the assessments reported herein, the answer to these questions will have to await further studies focused upon the mechanisms responsible for these changes in this endogenous antioxidant. Despite these

experimental considerations, this study clearly demonstrated that increased mitochondria-derived free radicals with ageing lead to endothelial dysfunction and this can be normalized by scavenging mitochondria-derived reactive oxygen species with MitoQ.

Conclusion

This study has demonstrated that, in human SMFAs, recognized to have regulatory potential, the attenuation of free radicals from the mitochondria in the vasculature with a mitochondria-targeted antioxidant reverses age-related vascular dysfunction by what appears to be an NO-dependent mechanism. These findings suggest that mitochondria-targeted antioxidants, such as MitoQ, may have utility in terms of counteracting the attenuated skeletal muscle blood flow and vascular dysfunction so often associated with advancing age and cardiovascular disease.

Methods

Subjects and general procedures

A total of 44 SMFAs were obtained from young and old subjects, from the axillary and inguinal regions, during melanoma-related surgeries. From these SMFAs, endothelial-dependent and endothelial-independent vascular functions were assessed in 10 young subjects, while 16 old subjects were assessed with and without MitoQ (Antipodean Pharmaceuticals, Inc., Menlo Park, CA, USA; gifted by M.P.M.). A subset of these vessels ($n = 8$ young and 8 old subjects) were assessed for mitochondria-specific O_2^- levels. Endothelial-dependent vascular function was assessed in the SMFAs from the remaining eight young subjects, while the remaining 10 old subjects were assessed with and without MitoQ and N$^\omega$-nitro-L-arginine methyl ester (L-NMMA). Unused segments of these vessels ($n = 8$ young and 10 old subjects) were used for immunoblotting. It should be noted that, although all subjects were free from cancer and chemotherapy, there were no other specific exclusion criteria for this study. However, all medical conditions and medications were noted. All protocols were approved by the Institutional Review Boards of the University of Utah and Salt Lake City

Veteran's Affairs Medical Center (VAMC), carried out in accordance with the Declaration of Helsinki, and written informed consent was obtained from all subjects prior to surgery.

Vessel harvest and preparation

Skeletal muscle feed arteries (outer diameter approx. 500 µm, length 1–2 cm) 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 VAMC, were studied. Patients were anaesthetized using a general protocol: propofol, fentanyl, benzodiazepines and succinylcholine.[40] SMFAs were harvested during dissection to locate sentinel lymph nodes, for clinical analysis, and were identified and classified based upon being a vascular inlet into a muscle bed, structure, coloration and pulsatile bleed pattern.[26] SMFAs were ligated, excised and immediately placed in ice-cold PSS before being transferred to the laboratory within 15 min of harvesting.[7]

MitoQ treatment and vessel function protocols

Initially, perivascular adipose and/or connective tissue around the SMFAs was removed under a dissecting microscope (SZX10; Olympus, Center Valley, PA, USA) in cold (4 °C) PSS containing (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 buffer and 10 g L^{-1} of BSA at pH 7.4. SMFA function was assessed in pressure myography organ baths (110p; DMT Systems, Aarhus, Denmark).[7] The arteries were cannulated at both ends with micropipette tips and then pre-incubated for 1 h within the bath in either PSS, the control condition or MitoQ (10 IM). After the pre-incubation period, the vessel outer diameters were recorded using an inverted microscope with a video camera (TS100; Nikon Eclipse, Melville, NY, USA), with data streamed in real time to edge detection software (DMT VAS v 0.2.0), monitored at a sampling rate of 1 kHz. Fluid leak was detected by pressurizing the vessel to an intra- luminal pressure set of 60 mmHg, closing the cannulas to the fluid

reservoirs and assessing any change in vessel diameter. Arteries free from leaks were then warmed to 37 °C, allowed to develop spontaneous tone for a 30- min equilibration period, and then vasodilator function was assessed.[7]

Vasodilation assessments

Vasodilator dose–response curves (%) were assessed for three stimuli: first, to assess the endothelium-dependent vasodilator response to flow-induced shear stress, intraluminal flow was developed. This was achieved by altering the heights of the independent fluid reservoirs, contiguous with both cannulated ends of 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 45 mmHg, which yielded an approximate flow rate of 15, 30 and 45 μL min^{-1}, were utilized for the flow experiments. Second, to assess endothelium-dependent vasodilation pharmacologically, an ACh dose–response curve (ACh, 10^{-7} to 10^{-3} M; Sigma-Aldrich Corp., St Louis, MO, USA) was performed following pre-constriction with phenylephrine (PE) (10^{-6} to 10^{-4} M; Sigma-Aldrich Corp.) to approx. 70% of the maximum PE response. Third, to assess endothelium-independent vasodilation, a SNP dose–response curve was performed (10^{-9} to 10^{-4} M) following pre-constriction with PE (10^{-6} to 10^{-4} M) to approx. 70% of the maximum PE response.

Mitochondria-specific O_2^- measurements

Mitochondria-specific O_2^- measurements were taken with EPR spectroscopy on the initially frozen SMFA segments using an EMX X-band spectrometer (Bruker, Billerica, MA, USA). Briefly, the segment of the frozen SMFA was placed into a microcentrifuge tube containing 150 μL of the mitochondria-specific O$^-$ spin trap mitoTempo-H (Enzo Life Sciences, San Diego, CA, USA) (1-hydroxy-4 [2-(triphenylphosphoino)-aceta- mido]-2,2,6,6-tetramethylpiperidine) (0.5 mmol L^{-1}) and incubated for 60 min at 37 °C, facilitating the 'thaw and trap' approach.[41,42] The samples were then placed on ice and 50 μL of the solution was loaded into a capillary tube for EPR spectroscopy analysis. The EPR spectroscopy scan

was run with a centre field at approx. g = 2.004, and the area under the curve of the spectra was calculated by double integration.[7]

Percentage vasodilation calculations. Percentage vasodilation was used for data expression to account for baseline differences in vessel diameter, and calculated using the following equation:

$$(DT - Dp / Di - Dp) \times 100,$$

where DT is the recorded diameter at a given time point, Dp is the diameter recorded after the addition of the vasoactive agent (i.e. pre-constriction diameter), and Di is the diameter recorded immediately before the addition of the vasoactive agent (initial diameter).

Immunoblotting

The relative abundance of proteins for the electron transport chain complexes, p- and eNOS and MnSOD was determined in SMFAs using western blot analysis. Briefly, SMFAs were homogenized in lysis buffer, supplemented with a protease/phosphate inhibitor cock- tail [10 µM sodium fluoride and 1 mM phenyl methyl sulphonyl fluoride (PMSF)] (Santa Cruz Biotech, Santa Cruz, CA, USA). Protein concentration was determined using the Bradford technique. 50 µg of homogenate was separated by polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and incubated with primary and secondary antibodies directed against the proteins of interest. Membranes were imaged on a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) and quantified with Image Lab software (Bio-Rad). The specific antibodies used to detect SMFA proteins included total OXPHOS Human Western Blot Antibody Cocktail (ab110411; Abcam, Cambridge, MA, UK), total eNOS (610296; BD Transduction, San Jose, CA, USA), p-eNOS at Ser1177 (9570; Cell Signaling, Boston, MA, USA) and superoxide dismutase 2 (SOD2) (SC-515068; Santa Cruz Biotech, Santa Cruz, CA, USA). The abundance of each protein was normalized to beta-actin (ab8227; Abcam, Cambridge, MA, UK), which served as a loading control.

Statistical analyses. The statistical analyses were per- formed using GraphPad Prism 7 software (La Jolla, CA, USA). Two-way repeated-measures ANOVA was used to assess changes in vessel diameter with and without MitoQ in response to flow, ACh and SNP. Two-way repeated-measures ANOVA was used to assess changes in vessel diameter with and without MitoQ and with and without L-NMMA in response to flow and ACh. When necessary, a Tukey's post hoc test was used to identify significant differences. For all other comparisons, one-way ANOVA was used to assess the group and, if necessary, a Tukey's post hoc test was used to identify the significant differences. For all analyses, a *P*-value of <0.05 was considered significantly different. All data are expressed as mean ± SEM.

Conflict of interest

Author contributions

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