

Mitochondrial oxidative stress and antioxidant therapy in arterial aging

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Integrative Physiology
2016

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IACUC protocol # 1105.06 and 1404.02

ABSTRACT

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Mitochondrial oxidative stress and antioxidant therapy in arterial aging

Thesis directed by College Professor of Distinction Douglas R. Seals

Advancing age is a primary risk factor for cardiovascular diseases (CVD), due primarily to development of age-related arterial dysfunction, including arterial endothelial dysfunction and stiffening of large elastic arteries. A key cellular mechanism underlying age-related arterial dysfunction is oxidative stress, a state in which cellular production of reactive oxygen species (ROS) such as superoxide exceeds endogenous antioxidant defense capabilities.

Vascular mitochondria are emerging as critical regulators of arterial function. Vascular mitochondria produce physiological levels of ROS (mtROS) for signaling, but mitochondrial dysfunction is characterized by excessive mtROS production. Thus, vascular mitochondria represent a key potential source of arterial oxidative stress; however, the role of mtROS in age-related arterial dysfunction has been unknown. Accordingly, the purpose of this dissertation was to determine the role of mitochondria-derived oxidative stress in arterial aging and to investigate the therapeutic potential of a mitochondria-targeted antioxidant, MitoQ, to ameliorate age-related arterial dysfunction.

In arteries of old mice, mitochondrial superoxide production was ~3 times greater than in arteries of young mice, and this was associated with arterial endothelial dysfunction, measured as a reduction in nitric oxide-mediated endothelium-dependent dilation (EDD). Acute, ex-vivo application of MitoQ to reduce mitochondrial oxidative stress abolished the age-associated impairment in EDD. Moreover, chronic, in vivo MitoQ supplementation (4 weeks in drinking water) in old mice completely restored EDD to levels similar to those of young mice,

accompanied by normalization of age-related alterations in protein markers of mitochondrial health measured in whole arteries.

Arterial aging in mice was also characterized by elevated large-elastic artery stiffness, assessed in vivo as aortic pulse-wave velocity (aPWV). MitoQ supplementation in old mice reduced aPWV to levels similar to those of young mice and this was at least partially mediated by attenuation of the age-related reduction in arterial elastin content.

Together, these results indicate that mitochondrial oxidative stress is a key mechanism underlying age-related arterial dysfunction. These studies demonstrate that reducing mitochondrial oxidative stress with the targeted antioxidant MitoQ restores EDD and reduces arterial stiffness in old mice, underscoring the therapeutic potential for mitochondria-targeted strategies to reduce mitochondrial oxidative stress, improve arterial function, and reduce CVD risk in humans.

ACKNOWLEDGEMENTS

When it comes to raising a scientist, it really might take a village—this was definitely true for my doctoral training, and so I would like to thank all the people who contributed not only to the completion of these research projects, but to my scientific and personal development.

Thanks to my committee members, Drs. Byrnes, Mazzeo, Link, and Chicco, for guidance throughout my doctoral training. Thanks also to Dr. Chicco for sharing your expertise on mitochondrial function and for being so willing to collaborate on new projects. A special thank you to my mentor, Dr. Seals, for invaluable scientific and professional training; for helping identify and acquire the resources necessary to perform my research projects; for sharing insights and wisdom from your years of experience; for challenging me to develop new skills; and for holding me to an impeccably high standard of quality in all of my work.

Thanks to all of the members of the Integrative Physiology of Aging Laboratory who were involved in my projects. These studies would not have been possible without the intellectual and technical contributions of so many people. Thanks also for the friendships (and many moments of laughter) that can only be developed in a research laboratory setting—it has been a pleasure to work with you all. Thanks also to the university laboratory animal care staff for their contributions to all of my work.

Thank you to Dr. Russ Moore and the members of the Moore lab, who provided me with such an overwhelmingly positive first exposure to research—it was this experience that inspired (and prepared) me to pursue doctoral training in the first place.

Finally, thanks to my family for unwavering love and support throughout this and all my other endeavors. Thanks to Ben for walking this road beside me.

Chapter

I.	Introduction.....	1
II.	Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice.....	5
	Abstract.....	5
	Introduction.....	7
	Methods.....	9
	Results.....	13
	Discussion.....	16
III.	Mitochondria-targeted antioxidant therapy with MitoQ ameliorates large elastic artery stiffness in old mice.....	31
	Abstract.....	31
	Introduction.....	33
	Methods.....	35
	Results.....	40
	Discussion.....	42
IV.	Voluntary aerobic exercise increases arterial resilience and mitochondrial health with aging in mice.....	51
	Abstract.....	51
	Introduction.....	53
	Methods.....	55
	Results.....	61
	Discussion.....	65
V.	Conclusion.....	84
VI.	References.....	87

LIST OF TABLES

Table II-1. General morphological characteristics and MitoQ intake.....	23
Table III-1. General morphological characteristics and blood pressure.....	46
Table IV-1. Select morphological characteristics and voluntary running wheel activity.....	76
Table IV-2. Protein expression of mitochondrial health markers in carotid arteries versus aorta of young and old mice	77
Table IV-3. Aortic protein expression of mitochondrial respiratory complex proteins.....	78

LIST OF FIGURES

Figure II-1. MitoQ reverses the age-related decline in endothelium-dependent dilation.....	24
Figure II -2. MitoQ restores nitric oxide-dependent endothelium-dependent dilation in old mice.....	25
Figure II -3. MitoQ normalizes vascular oxidative stress in old mice.....	26
Figure II -4. MitoQ reduces arterial mitochondria-derived oxidative stress and suppression of function in old mice.....	27
Figure II -5. MitoQ improves markers of mitochondrial health in old mice.....	28
Figure II -6. MitoQ improves resistance to acute mtROS stress in arteries of old mice.....	29
Figure II -7. Working hypothesis.....	30
Figure III-1. MitoQ treatment reverses age-related arterial stiffness in mice.....	47
Figure III-2. MitoQ treatment attenuates the age-related decline in elastin-mediated intrinsic mechanical properties but has no effect on collagen-mediated intrinsic mechanical stiffness.....	48
Figure III-3. MitoQ treatment attenuates the age-related reduction in aortic elastin expression..	49
Figure III-4. MitoQ treatment in old mice reduces aortic MMP-2 expression.....	50
Figure IV-1. Voluntary aerobic exercise restores endothelium-dependent dilation and normalizes mitochondrial superoxide production in old mice.....	79
Figure IV-2. Voluntary aerobic exercise increases arterial resilience to acute mitochondria-specific stress.....	80
Figure IV-3. Voluntary aerobic exercise increases arterial resilience to acute simulated Western diet stress.....	81
Figure IV-4. Voluntary aerobic exercise restores markers of arterial mitochondrial health in old mice.....	82
Figure IV-5. Voluntary aerobic exercise augments arterial markers of antioxidant defense and stress resistance.....	83

CHAPTER I INTRODUCTION

Advancing age is the primary risk factor for cardiovascular diseases (CVD), which remain the leading cause of morbidity, disability and death in developed societies (Lloyd-Jones et al., 2010; Roger et al., 2012). The primary cause of increased CVD risk with age is adverse changes to arteries, specifically the development of arterial endothelial dysfunction and stiffening of the large elastic arteries (Herrera et al., 2010; Lakatta & Levy, 2003; Najjar et al., 2005; Seals et al., 2014; Yeboah et al., 2007). Indeed, declines in endothelial function, assessed as reduced endothelium-dependent dilation (EDD), and increases in arterial stiffness, assessed as elevated aortic pulse-wave velocity (aPWV), are independent predictors of CVD risk (Mitchell et al. 2010, Vita & Kearney, 2002; Yeboah et al., 2007). Demographic trends project a new “epidemic” of clinical CVD in coming decades, driven largely by increases in the number of middle-aged and older adults, that—in the absence of intervention—will become an enormous biomedical and societal burden (Heidenreich et al., 2011; Olshansky et al., 2009). Thus, identifying strategies that preserve arterial function and reduce CVD risk in this population is an important biomedical goal.

A primary cellular mechanism underlying age-related arterial dysfunction is oxidative stress, a state characterized by increased production of reactive oxygen species (ROS) relative to cellular antioxidant defenses. Oxidative stress impairs endothelial function by decreasing the bioavailability of the critical vasodilatory and vasoprotective molecule nitric oxide (NO), and also promotes overall disruption of cellular homeostasis, including induction of adverse pro-inflammatory signaling that suppresses vascular endothelial function (Bachschmid et al., 2013; Brandes et al., 2005; Lakatta, 2003; Seals et al., 2014; van der Loo et al., 2000). Oxidative stress also contributes to the development of age-associated arterial stiffness by inducing structural

changes to arteries, including increases in the load-bearing and stiffness-instilling protein collagen, degradation of the flexibility-instilling protein elastin, and formation of cross-links among proteins that further increase stiffness (Greenwald, 2008; Lakatta, 2003; Ziemann et al., 2005).

Because the persistent cellular production of excessive ROS plays such a central role in age-related arterial dysfunction, reducing oxidative stress seems to be a particularly promising therapeutic strategy. However, despite strong evidence that oxidative stress is a key mechanism mediating impaired arterial function with age, clinical trials with traditional exogenous antioxidants have proven largely unsuccessful (Drummond et al., 2011; Eskurza et al, 2004a; Eskurza et al., 2004b; Kris-Etherton et al., 2004). This lack of efficacy may be due in part to inefficient uptake of general antioxidants into cells, such that adequate levels of these compounds may be unable to reach the cellular sources of ROS at concentrations that reduce or prevent their adverse effects. Additionally, most general antioxidant compounds have short half-lives (e.g., 30 minutes) that prevent sustained beneficial effects (Drummond et al., 2011; Murphy, 2014).

The recent development of mitochondria-targeted antioxidants offers a promising, novel therapeutic strategy for reducing vascular oxidative stress and resulting functional sequelae, while avoiding these limitations of general, exogenous antioxidant treatments (Lakshminarasimhan & Steegborn, 2011; Smith & Murphy, 2010; Murphy, 2014). Mitochondria are crucial for maintaining cellular homeostasis and signaling functions in the vasculature, many of which are mediated by the production of ROS (mtROS), primarily superoxide, at physiological levels (Dai et al., 2012; Dromparis & Michelakis, 2013; Kluge et al., 2012; Quintero et al., 2006; Widlansky & Gutterman, 2011). However, mitochondria become

dysfunctional with aging and produce excessive levels of mtROS that are the central feature of a ‘vicious cycle’ of mitochondrial oxidative stress and dysfunction. Consequences of elevated mtROS include direct oxidative damage to mitochondrial components that promotes further impairments in mitochondrial respiration, metabolism, biogenesis, dynamics (e.g., increased fission/reduced fusion) and antioxidant defenses, as well as activation of other cellular pro-oxidant and stress response pathways (James & Murphy, 2002; Lopez-Armada et al., 2013; Mammucari & Rizzuto, 2010; Quintero et al., 2006; Seo et al., 2010; Weber & Reichert, 2010). Given the potential widespread effects of excessive mtROS, mitochondrial oxidative stress may represent a novel therapeutic target for improving or preserving arterial function with aging.

Mitoquinone (MitoQ) is a mitochondria-targeted antioxidant compound composed of the naturally-occurring antioxidant ubiquinone/coenzyme Q₁₀ conjugated to a lipophilic cation (triphenylphosphonium [TPP]). The lipophilicity and positive charge of this cation help drive MitoQ to the inner mitochondrial membrane, where it accumulates at levels 100-1,000-fold higher than in the cytosol of cells (Cocheme et al., 2007; Ross et al., 2008; Smith & Murphy, 2010). Although traditional, untargeted antioxidants (which are dispersed throughout many cellular locations) may interact with some mtROS, the addition of the mitochondria-targeting moiety optimally positions MitoQ at a major site of mtROS production to reduce mitochondrial oxidative stress. MitoQ is also a recyclable antioxidant; the active (reduced) form of MitoQ is regenerated by reaction with mitochondrial respiratory complex II (succinate-coenzyme Q reductase), allowing for sustained antioxidant activity (Ross et al., 2008; Smith & Murphy, 2010). MitoQ improves physiological function in pre-clinical models of clinically-relevant diseases (Mercer et al., 2012; Miquel et al., 2014; reviewed in Smith & Murphy, 2010), has been used safely in Phase II clinical trials for Parkinson’s and liver disease (Gane et al., 2010; Snow et al.,

2010) and has recently become commercially available as a dietary supplement. However, there has been no information about the potential for MitoQ to ameliorate arterial dysfunction associated with primary aging. Accordingly, in Chapters II and III of this dissertation we investigated the therapeutic potential of MitoQ supplementation to improve arterial endothelial function (Chapter II) and reduce arterial stiffness (Chapter III) in aged mice, with the goal of establishing evidence and rationale to perform clinical trials in older adults.

In addition to baseline deficits in function, another hallmark of arterial aging is reduced resilience, i.e., the ability to withstand stress. Indeed, cross-sectional data in humans indicate that age-related impairments in endothelial function are exacerbated in the presence of common risk factors such as elevated blood glucose or LDL cholesterol (deVan et al., 2013; Walker et al., 2009). Feeding mice a “Western”-style diet (WD) high in saturated fat and sugar causes impairments in arterial function, but the magnitude of impairment induced by WD feeding is greater in old mice compared to young mice (Lesniewski et al., 2013), also supporting the premise of reduced arterial resilience with advancing age.

Mitochondria are critical components of cellular stress response (Galuzzi et al., 2012; Manoli et al., 2013; Nunnari & Suomalainen, 2012), but declines in mitochondrial health with advancing age may contribute to reduced resilience, as many common stressors to which aging arteries are exposed, including high circulating levels of fatty acids and glucose, induce cellular production of mtROS (Gao et al., 2011; Koziel et al., 2015; Lu et al., 2013; Makino et al., 2010; Shenouda et al., 2011; Yuzefovych et al., 2010). Thus, in Chapter IV, we examined the effects of aging on arterial resilience to acute mtROS stressors and also investigated the potential for late-life aerobic exercise—an intervention that improves mitochondrial health (Hood et al., 2011; Koltai et al., 2012)—to restore arterial resilience.

CHAPTER II

Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice

Gioscia-Ryan RA, LaRocca TJ, Sindler AL, Zigler MC, Murphy MP, Seals DR. *J Physiol*, **592(12)**, 2549-61, 2014.

ABSTRACT

Age-related arterial endothelial dysfunction, a key antecedent to the development of cardiovascular diseases (CVD), is largely due to a reduction in nitric oxide (NO) bioavailability as a consequence of oxidative stress. Mitochondria are a major source and target of vascular oxidative stress when dysregulated. Mitochondrial dysregulation is associated with primary aging, but its role in age-related endothelial dysfunction is unknown. Our aim was to determine the efficacy of a mitochondria-targeted antioxidant, MitoQ, for ameliorating vascular endothelial dysfunction in old mice. *Ex vivo* carotid artery endothelium-dependent dilation (EDD) to increasing doses of acetylcholine was impaired ~30% in old (~27 mo.) compared to young (~8 mo.) mice due to reduced NO bioavailability ($p < 0.05$). Acute (*ex vivo*) and chronic (4 weeks in drinking water) administration of MitoQ completely restored EDD in older mice by improving NO bioavailability. There were no effects of age or MitoQ on endothelium-independent dilation to sodium nitroprusside. The improvements in endothelial function with MitoQ supplementation were associated with normalization of age-related increases in total and mitochondria-derived arterial superoxide production and oxidative stress (nitrotyrosine abundance), as well as increases in markers of vascular mitochondrial health, including antioxidant status. MitoQ also reversed the age-related increase in endothelial susceptibility to acute mitochondrial damage (rotenone-induced impairment in EDD). Our results suggest that mitochondria-derived oxidative stress is an important mechanism underlying the development of endothelial dysfunction with primary aging. Mitochondria-targeted antioxidants such as MitoQ represent a promising, novel

strategy for preserving vascular endothelial function with advancing age and preventing age-related CVD.

INTRODUCTION

Advancing age is the primary risk factor for cardiovascular diseases (CVD) (Roger et al., 2012), driven significantly by the development of arterial endothelial dysfunction (Herrera et al., 2010; Yeboah et al., 2007). Impaired endothelium-dependent dilation (EDD), largely a result of reduced nitric oxide (NO) bioavailability secondary to increased oxidative stress (Bachschmid et al., 2013; Lakatta, 2003; van der Loo et al., 2000), is an important clinical manifestation of endothelial dysfunction.

Oxidative stress, a state in which the production of pro-oxidant molecules such as superoxide outweighs endogenous antioxidant defense mechanisms, directly and indirectly reduces NO bioavailability. Superoxide reacts with NO to form peroxynitrite, which, in turn, can oxidize and inactivate tetrahydrobiopterin, a necessary co-factor for endothelial NO synthase (eNOS). As a result, eNOS becomes uncoupled and produces additional superoxide rather than NO (Brandes et al., 2005; van der Loo et al., 2000). Peroxynitrite also propagates oxidative stress via excessive nitration of cellular proteins, including the key antioxidant enzyme manganese superoxide dismutase (MnSOD), effectively reducing endogenous antioxidant capacity (Brandes et al., 2005; van der Loo et al., 2000). Overall, the age-related increase in oxidative stress represents a vicious cycle of processes that interact to reduce NO and impair endothelial function.

Healthy mitochondria are crucial for maintaining numerous aspects of physiological function and mitochondrial reactive oxygen species (mtROS), primarily produced as superoxide and subsequently converted to hydrogen peroxide, play a crucial role in cellular signaling and maintenance of homeostasis in the vasculature (Dai et al., 2012; Dromparis & Michelakis, 2013; Kluge et al., 2013; Quintero et al., 2006). However, excessive mtROS production leads to a state of cellular oxidative stress and is a hallmark of mitochondrial dysfunction (Dromparis &

Michelakis, 2013; Murphy, 2009). Excess mtROS may promote disruption of vascular function directly (via oxidative stress) and indirectly through mitochondrial dysfunction-induced alterations in signaling (Dai et al., 2012; Dromparis & Michelakis, 2013; Kluge et al., 2013). Mice with genetic MnSOD deficiency, a model of elevated mitochondrial oxidative stress, display impairments in endothelial function (Wenzel et al., 2008). However, the role of mtROS in primary aging-associated vascular endothelial dysfunction has not been established.

Compounds that specifically target mtROS hold great promise for ameliorating impairments, including vascular dysfunction, that stem from mitochondria-associated oxidative stress (Cochemé et al., 2007; Lakshminarasimhan & Steegborn, 2011; Murphy & Smith, 2007; Smith et al., 2012). Because mitochondrial oxidative stress is closely linked to overall mitochondrial health, reducing mitochondrial oxidative stress may have the potential to restore homeostasis in this critical cellular organelle and, in turn, improve cellular homeostasis and physiological function. In contrast to traditional non-targeted exogenous antioxidants, which have been ineffective in clinical trials (Kris-Etherton et al., 2004), mitochondria-targeted antioxidants accumulate specifically at this site of ROS production and may, therefore, be more effective at reducing oxidative stress and resulting functional sequelae (Cochemé et al., 2007; Smith et al., 2012). One such compound is mitochondria-targeted ubiquinone, or MitoQ. MitoQ is a biochemically modified form of the naturally-occurring antioxidant ubiquinone conjugated to a lipophilic cation (decyl-triphenylphosphonium [TPP]) (Murphy & Smith, 2007; Smith & Murphy, 2010). The positive charge and lipophilicity of this cation drive MitoQ to accumulate predominately in the inner mitochondrial membrane (Ross et al., 2008), where it blocks mitochondrial oxidative damage. The reduced form of MitoQ is then regenerated via reaction with mitochondrial respiratory complex II (succinate-coenzyme Q reductase) (Ross et al., 2008;

Smith & Murphy, 2010). MitoQ has been studied in models of CVD and in patients with clinical disease, but the efficacy of MitoQ for reversing primary arterial aging, including vascular endothelial dysfunction, is unknown.

Here, we tested the hypothesis that oral MitoQ treatment would ameliorate vascular endothelial dysfunction in old mice, and that these improvements would be associated with reductions in vascular mitochondrial oxidative stress and improvements in vascular mitochondrial health.

METHODS

Ethical approval

All studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder and conform to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Animals

Male c57BL/6 mice, an established model of age-related vascular endothelial dysfunction (Brown et al., 2007; Sprott & Ramirez, 1997), were purchased from the aging colony at the National Institute on Aging at ~6 or ~25 months of age and allowed to acclimate to our facilities for 2 weeks prior to beginning treatment. Mice were housed in standard cages on a 12-hour light/dark cycle and were allowed access to normal rodent chow (Harlan 7917) and water ad libitum. Body mass and water intake were monitored regularly throughout the study.

MitoQ treatment

Based on previous findings of effective dose and duration of treatment of MitoQ (Rodriguez-Cuenca et al., 2010; Smith & Murphy, 2010), mice were randomly assigned to treatment with MitoQ (250 μ M) (young MitoQ-treated [YMQ, ~8 mo., n=6] and old MitoQ-

treated [OMQ, ~27 mo., n=14]) or normal drinking water (young control [YC, ~8 mo., n=12] and old control [OC, ~27 mo., n=13]) for 4 weeks. MitoQ (Antipodean Pharmaceuticals, gift from MPM) was prepared fresh and administered in light-protected water bottles changed every three days. To rule out potential effects of the TPP cation (mitochondria-targeting moiety), additional groups of young and old mice were provided drinking water containing a control compound comprising only decyl-TPP cation (n=5-6/group, YMP and OMP) and not the antioxidant (Adlam et al., 2005; Graham et al., 2009; Smith & Murphy, 2010).

Vascular endothelial function

Following the 4-week treatment period, mice were anesthetized with isoflurane and killed by exsanguination via cardiac puncture. EDD and endothelium-independent dilation (EID) were measured in isolated carotid arteries as previously described (LaRocca et al., 2013; Rippe et al., 2010). The carotid arteries were dissected free of surrounding tissue and cannulated onto glass micropipettes in warmed (37° C) physiological saline solution in myograph chambers (DMT, Aarhus, Denmark). Arteries were pressurized to 50 mmHg and allowed to equilibrate for ~1h prior to the beginning of experiments. Following precontraction with phenylephrine (2 μ M, Sigma Aldrich, St. Louis, MO, USA), EDD was assessed by measuring the increase in luminal diameter in response to increasing concentrations of acetylcholine (ACh, 1×10^{-9} to 1×10^{-4} M, Sigma Aldrich). EID was measured as dilation in response to increasing doses of the exogenous NO donor sodium nitroprusside (SNP, 1×10^{-10} to 1×10^{-4} M, Sigma Aldrich). To account for baseline differences in vessel diameter, all dose-response data are reported on a percent basis.

NO-mediated EDD. EDD was assessed in the presence of the eNOS inhibitor N-Nitro-L-Arginine Methyl Ester (L-NAME, 0.1 mM, 30 minute incubation, Sigma Aldrich) and the

contribution of NO was calculated as the difference between maximal dilation to ACh alone and maximal dilation in the presence of L-NAME (LaRocca et al., 2012; Rippe et al., 2010).

Chronic mtROS suppression of EDD. To determine tonic mtROS suppression of EDD, arteries were incubated for 40 minutes with 1 μ M MitoQ to scavenge mtROS prior to assessment of EDD to ACh as described above.

Acute mtROS challenge. To determine the effects of an acute increase in mtROS on EDD, a subset of arteries were incubated for 40 minutes with 0.5 μ M rotenone (Sigma Aldrich), a concentration previously shown to stimulate mtROS production at respiratory Complex I without completely inhibiting cellular respiration (Li et al., 2003; Weir et al., 1991) prior to assessment of EDD to ACh. The difference between maximal dilation to ACh in the presence vs. absence of rotenone was calculated to determine the rotenone-induced decrement in EDD.

Aortic whole-cell and mitochondria-specific superoxide production

Measurement of superoxide production in the thoracic aorta was performed via electron paramagnetic resonance spectroscopy, as described previously (Fleenor et al., 2012; LaRocca et al., 2012; LaRocca et al., 2013). Briefly, the aorta was removed and dissected free of surrounding tissue. 2 mm segments were incubated for 1 hour at 37 °C in Krebs-HEPES buffer with the superoxide-specific spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (0.5 mM, Enzo Life Sciences, Farmington, NY, USA) or mitochondrial superoxide-specific spin probe MitoTEMPO-H (0.5 mM, Enzo Life Sciences) for detection of whole-cell and mitochondria-specific superoxide production, respectively (Dikalov, et al., 2011; Dikalova et al., 2010). The signal amplitude was analyzed using an MS300 X-band EPR spectrometer (Magnettech, Berlin, Germany) with the following settings: centerfield, 3350 G;

sweep, 80 G; microwave modulation, 3000 mG; microwave attenuation, 7 dB. Data are presented relative to the mean of the young control group.

Aortic protein expression

Protein expression was determined by standard Western blotting techniques using segments of thoracic aorta, a representative large elastic artery that, unlike the carotid, provides sufficient sample for analysis (LaRocca et al., 2012; Rippe et al., 2010). Following homogenization in radio-immunoprecipitation assay lysis buffer, 15 µg of aortic protein were loaded onto 4-12% polyacrylamide gels and transferred onto nitrocellulose membranes (Criterion System; Bio-Rad, Hercules, CA, USA). Membranes were incubated (overnight at 4° C) with primary antibodies: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, normalizer, Cell Signaling, Boston, MA, USA), phosphorylated (ser36) p66^{SHC} (1:500, Abcam, Cambridge, MA, USA), cytochrome c oxidase (COX-IV, 1:1000, Cell Signaling), MnSOD (1:2000, Enzo Life Sciences), nitrotyrosine (NT, 1:500, Abcam) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α, 1:1000, Cell Signaling). Proteins were visualized on a digital acquisition system (ChemiDoc-It, UVP, Upland, CA, USA) using chemilluminescence with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Westgrove, PA, USA) and ECL substrate (Pierce, Rockford, IL, USA). Relative intensity was quantified using ImageJ software and normalized to GAPDH intensity and then expressed as a ratio of the mean intensity of the young control group.

Nitric oxide synthase activity assay

Activity of nitric oxide synthase (NOS) was determined in aortic lysates using the Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase (Oxford Biomedical Research, Oxford, MI, USA), according to the manufacturer directions. Briefly, 30 µL of aortic lysate

(containing 40-60 μg protein) were incubated with NOS substrates NADPH and L-arginine for 4 hours to allow continual production of NO by NOS. This reaction was carried out in aqueous solution, in which NO rapidly degrades into the more stable products nitrate and nitrite. Nitrate reductase was added to the samples to facilitate the enzymatic conversion of nitrate to nitrite. Subsequently, nitrite (representing the total NO generated by NOS) was quantified colorimetrically using Griess reagent. The concentration of nitrite in each sample was determined by interpolating from a standard curve generated using known concentrations of nitrite. Values were normalized to the protein content of each sample and expressed as μmoles of NO produced per μg of aortic protein.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 software (Armonk, NY, USA) with an alpha value of 0.05. EDD and EID dose-responses to ACh and SNP, respectively, were analyzed using 2-factor (treatment group x dose) repeated measures ANOVA. Within-group differences in EDD dose-responses to ACh in the absence vs. presence of pharmacological modulation (e.g., L-NAME, rotenone) were also determined using 2-factor (condition x dose) repeated measures ANOVA. For all other outcomes, group differences were determined using one-way ANOVA. When a significant main effect was observed, Tukey's honestly significant difference post-hoc tests were performed to determine specific pair-wise differences.

RESULTS

Animal characteristics and MitoQ intake

Selected morphological characteristics and water intake are shown in **Table 1**. There were no differences in body mass across groups, and organ weights did not differ between

control and MitoQ-treated mice, indicating an absence of off-target effects. MitoQ intake was similar between young and old treated groups.

MitoQ treatment reverses the age-related decline in EDD

Primary comparison. Carotid artery dose response (**Figure 1A**) and peak (**Figure 1C**) EDD to ACh were reduced in old compared to young mice. *In vivo* (4 weeks) MitoQ supplementation restored EDD in old mice (**Figure 1A and C**).

Control comparisons. MitoQ treatment had no effect on EDD in young mice (**Figure 1B and C**) and EDD did not differ in young or older control (normal drinking water) vs. decyl-TPP treated mice (**Figure 1B and C**).

There were no differences in endothelium-independent dilation in response to the NO donor SNP among the groups (**Figure 1D**). These results indicate that short-term treatment with MitoQ restores ACh-mediated endothelial function with aging in mice. Because the normal drinking water and decyl-TPP treated groups did not differ, all subsequent analyses were performed only in the normal drinking water and MitoQ treated groups (YC, OC, YMQ and OMQ) in order to increase statistical power.

MitoQ treatment restores NO bioavailability in old mice

The reduction in EDD in old mice was a result of reduced NO bioavailability, as indicated by a lesser reduction in EDD upon incubation with the eNOS inhibitor L-NAME (**Figures 2A and B**). MitoQ supplementation normalized the NO component of EDD in old mice while having no effect in young mice (**Figure 2B**). These results indicate that MitoQ increases NO bioavailability and restores the NO-component of EDD in old mice.

Total NOS activity was reduced in arteries of old compared to young mice, and this was not altered by MitoQ supplementation (**Figure 2C**), indicating that the restoration of NO bioavailability with MitoQ treatment was not the result of changes in eNOS activity.

MitoQ supplementation normalizes vascular oxidative stress in old mice

Aorta from old mice exhibited greatly increased nitrotyrosine (**Figure 3A**), a biomarker of general oxidative protein modification (Radi, 2013; Radi, 2004), and markedly increased whole-vessel superoxide production (**Figure 3B**) compared to young mice. MitoQ treatment ameliorated the age-related increases in aortic nitrotyrosine and superoxide while having no effect in young mice. These data indicate that MitoQ has potent antioxidant effects in arteries of old mice, which could contribute to its beneficial effects on vascular endothelial function.

MitoQ supplementation reverses arterial mitochondria-derived oxidative stress and suppression of function in old mice

Aortic mitochondrial superoxide production was substantially greater in old compared to young mice (**Figure 4A**), as was aortic phosphorylated (activated) p66^{SHC} (**Figure 4B**), a signaling protein that is a marker and master regulator of mitochondrial oxidative stress (Gertz & Steegborn, 2010). MitoQ treatment in old mice ameliorated the age-related increases in mitochondrial superoxide and p-p66^{SHC}.

Acute (40 minutes) incubation of carotid arteries with MitoQ (1.0 μ M) restored EDD to ACh in old control animals, indicating excessive mitochondrial superoxide-mediated suppression of EDD with aging (**Figure 4C**). Acute MitoQ treatment had no effect on EDD in young control or young and old MitoQ treated animals.

Together, these results indicate that an increase in arterial mitochondrial superoxide production and mitochondrial oxidative stress contributes to age-related declines in endothelial

function. Moreover, the data suggest that MitoQ treatment markedly reduces vascular mitochondrial superoxide production and oxidative stress, which is associated with complete rescue of endothelial function in old mice.

MitoQ restores markers of vascular mitochondrial health in old mice

Protein expression of markers of mitochondrial signaling/biogenesis, antioxidant defense and mass (PGC-1 α , MnSOD and COX-IV) was reduced in arteries of old compared to young mice (**Figure 5 A-C**). MitoQ treatment restored these markers of mitochondrial health in old mice to levels similar to young controls, and further increased COX-IV expression in young mice. These results indicate that reducing mitochondrial oxidative stress with MitoQ treatment may also restore mitochondrial homeostasis in the aging vasculature.

MitoQ supplementation improves resistance to acute mtROS stress in arteries of old mice

Acute incubation with 0.5 μ M rotenone to stimulate production of mtROS (Li et al., 2003; Weir et al., 1991) caused a significant further (~25%) reduction in carotid artery EDD to ACh in old control mice, but had no significant effect on EDD in young control mice (**Figure 6**). MitoQ treatment attenuated the rotenone-induced impairment of EDD in old mice, while having no effect in young mice. These results indicate that aging is associated with reduced resistance to an acute mtROS challenge in arteries of mice, and that MitoQ treatment improves resistance to this mtROS stressor to levels observed in young mice.

DISCUSSION

Our results here demonstrate for the first time that a mitochondria-targeted antioxidant (MitoQ) completely reverses endothelial dysfunction in old mice, restores NO bioavailability and normalizes total and mitochondria-derived arterial superoxide production and oxidative stress (nitrotyrosine abundance). These improvements are accompanied by normalization of age-related

declines in markers of vascular mitochondrial health, and an increased ability of arteries to resist an acute stress induced by excessive mtROS.

The present results extend previous findings by our laboratory showing that restoring NO-bioavailability and reducing oxidative stress in old mice ameliorates endothelial dysfunction (Donato et al., 2013; Fleenor et al., 2013; LaRocca et al., 2013; Rippe et al., 2010; Sindler et al., 2011) by specifically examining the role of mitochondria-derived oxidative stress and dysregulation in mediating age-related endothelial dysfunction. Although it is well established that age-related declines in mitochondrial function and increases in mitochondria-derived oxidative stress contribute to the development of dysfunction in other tissues such as skeletal muscle, heart, and brain (Balaban et al., 2005; Sastre, 2003; Weber & Reichert, 2010), the role of vascular mitochondrial oxidative stress in mediating impairments in endothelial function with primary aging is unknown. Our results demonstrate that aging is associated with increases in mitochondria-specific superoxide production and protein markers of mitochondrial oxidative stress in the large elastic arteries of mice. Importantly, inhibiting mitochondrial oxidative stress with MitoQ either acutely (*ex vivo*) or chronically (*in vivo* supplementation) abolished the age-related reduction in EDD by restoring NO bioavailability secondary to a reduction in oxidative stress, and not by obvious improvement in eNOS enzyme activation or function. These observations provide strong evidence that excess mitochondrial oxidative stress is an important mechanism underlying the development of endothelial dysfunction with aging, supporting the efficacy of mitochondria-targeted strategies to improve endothelial function with aging.

Mitochondrial production of ROS has previously been implicated in the progression of vascular dysfunction in the settings of clinical CVD and in genetic models of mitochondrial antioxidant deficiency. Production of mtROS can be induced by exposing cultured endothelial

cells to adverse conditions associated with cardiometabolic disease (e.g., hyperglycemia) (Shenouda et al., 2011), and cross-sectional studies in humans and rodent models have shown that CVD is accompanied by increased vascular mitochondrial damage/dysfunction (Ballinger, 2002; Ungvari et al., 2008; Zhang & Gutterman, 2007). Endothelial function is also impaired in mice with genetic MnSOD insufficiency, a model of excess mitochondrial oxidative stress (Wenzel et al., 2008). Together, data in experimental and disease models indicate that excess mtROS play a critical role in mediating vascular dysfunction (Wenzel et al., 2008). However, the present data provide the first evidence that mtROS contribute to vascular endothelial dysfunction with primary aging.

Despite the relative paucity of mitochondria in the endothelium compared to tissues such as skeletal muscle and liver (Blouin et al., 1977), our results suggest a pivotal role of mitochondria-related signaling and mtROS in modulating endothelial function with age. This possibility is further supported by previous studies showing a life-extending effect of endothelial cell-specific knockout of p66^{SHC}, a signaling protein involved in sensing and regulation of mtROS production (Camici et al., 2007; Gertz & Steegborn, 2010). We observed a marked elevation in phosphorylation of p66^{SHC}, an indication of its activation (Gertz & Steegborn, 2010), in arteries of old compared to young mice, and this was accompanied by increased vascular mitochondrial superoxide production. MitoQ normalized p66^{SHC} activation and reduced mitochondrial superoxide production, suggesting that an increase mtROS-mediated vascular oxidative stress may be a key mechanism contributing to the age-related decline in endothelial function.

Our results also support a critical role for healthy mitochondria in the maintenance of vascular endothelial function with aging. Mitochondria are an important source of cellular ROS,

which are produced at several sites, including Complexes I, II, and III of the respiratory chain, enzymes involved in electron transfer and mitochondrial metabolism (e.g., α -ketoglutarate dehydrogenase, electron transfer flavoprotein:coenzyme-Q oxidoreductase), p66^{SHC}, the monoamine oxidase family of enzymes on the outer mitochondrial membrane, and nicotinamide adenine dinucleotide phosphate oxidase 4, which localizes to the mitochondria (Kluge et al., 2013; Murphy, 2009). Due to their role in production of and proximity to ROS, mitochondria are particularly vulnerable to oxidative damage (Murphy, 2009; Weber & Reichert, 2010). Healthy mitochondria are equipped with antioxidant defense systems that act to maintain mtROS at physiological levels and facilitate signaling functions (Nunnari & Suomalainen, 2012; Dromparis & Michelakis, 2013), but prolonged, uncontrolled oxidative stress can lead to inhibition of mitochondrial function and related signaling pathways, including a reduction in mitochondrial antioxidant enzymes and biogenesis (Anderson & Prolla, 2009; Dromparis & Michelakis, 2013; Galluzzi et al., 2012). Our findings indicate that the age-related increase in vascular mtROS is associated with disruption of vascular mitochondrial homeostasis, as we observed a decline in the mitochondrial antioxidant MnSOD in arteries of old mice as well as reductions in protein markers of mitochondrial biogenesis (PGC-1 α) and mass (COX-IV), all of which were restored with MitoQ treatment.

Mitochondria are critical for mediating the cellular response to oxidative stress, such as occurs cumulatively with advancing age (Galluzzi et al., 2012; Nunnari & Suomalainen, 2012; Sastre, 2003; Zhang & Gutterman, 2007). Our observation that arteries from old mice had impaired ability to resist an acute mtROS-induced stress (administration of rotenone) is indicative of age-related dysregulation of mitochondria, including a reduction in mitochondrial antioxidant defenses (e.g., MnSOD). This finding is consistent with previous work showing that

genetic MnSOD deficiency in mice aggravates age-associated endothelial dysfunction (Wenzel et al., 2008). Rotenone, a known inhibitor of mitochondrial respiratory Complex I, also stimulates mtROS production without completely inhibiting cellular respiration when applied at low concentrations ($<1 \mu\text{M}$; Li et al., 2003). Previous studies (Csiszar et al., 2006; Griffith et al., 1986; Rodman et al., 1991; Weir et al., 1991) examining the effects of mitochondrial respiratory inhibition on endothelial function have demonstrated impairment in EDD following rotenone administration (concentrations $\geq 1 \mu\text{M}$), but the magnitude of impairment differed among species and types of arteries tested. It is also plausible that complete inhibition of mitochondrial respiration may have distinctly different effects on vascular function than an increase in mtROS, perhaps explaining lack of significant impairment observed in our young mice, which—in contrast to old mice—are expected to have adequate mitochondrial antioxidant defenses to counter this acute stressor. Importantly, MitoQ treatment in old mice restored the ability of arteries to resist an acute increase in mtROS to levels similar to what was observed in young mice. Together, our data provide evidence that reducing mitochondrial oxidative stress in the vasculature (e.g., via MitoQ supplementation) may restore overall mitochondrial health, with corresponding functional improvements.

In support of the present observations on primary aging, MitoQ has also been reported to ameliorate dysfunction associated with mitochondrial oxidative damage in several animal models of clinical disease, including cardiac ischemia-reperfusion injury, sepsis, fatty liver disease, kidney disease, neurodegeneration, and CVD (reviewed in Smith & Murphy, 2010). Whereas MitoQ supplementation initiated prior to establishment of CVD in young (8 week-old) stroke-prone hypertensive rats prevented the development of endothelial dysfunction (Graham et al., 2009), our results here are the first to demonstrate the efficacy of MitoQ for reversing vascular

dysfunction in aged animals. MitoQ treatment has also been successful in Phase II clinical trials in patients with liver and neurological diseases (Gane et al., 2010; Snow et al., 2010), underscoring strong potential for translation to human vascular aging.

Although the observed changes in arterial mitochondrial superoxide production and protein expression of select mitochondrial markers strongly suggest age- and treatment-associated effects on mitochondrial health and homeostasis, future investigation is warranted to more fully characterize vascular mitochondrial function (e.g., respiratory function, ATP production, calcium signaling, biogenesis, fission/fusion dynamics) in this setting. It is well established that mtROS and mitochondrial dysfunction contribute to adverse cellular signaling, including activation of inflammatory pathways that may exacerbate endothelial dysfunction (López-Armada et al., 2013; Nunnari & Suomalainen, 2012; Ungvari et al., 2007), but the potential for mitochondrial antioxidant treatment to attenuate adverse inflammatory signaling in the vasculature is currently unknown. Our findings that MitoQ treatment not only reduces mitochondrial oxidative stress, but also restores markers of mitochondrial health in arteries of old mice indicate that this treatment may reduce age-related adverse signaling via restoration of mitochondrial homeostasis.

In conclusion, the present study demonstrates for the first time that a mitochondria-targeted antioxidant, MitoQ, effectively reverses age-related vascular endothelial dysfunction, restores NO bioavailability, normalizes total and mitochondria-derived oxidative stress, and improves vascular mitochondrial health and stress resistance. These results indicate that mitochondria-targeted antioxidants may represent a novel, promising strategy for preserving healthy vascular endothelial function with primary aging in humans, and preventing age-related CVD.

ACKNOWLEDGEMENTS

The authors would like to thank Micah Battson for his technical assistance.

FUNDING

This work was supported by the National Institutes of Health (AG000279, AG0379210, AG013038, HL107120-04).

Table 1. General morphological characteristics and MitoQ intake

	YC	OC	YMQ	OMQ
Body mass (g)	30.06 ± 2.15	30.38 ± 3.69	28.97 ± 0.80	29.51 ± 1.54
Heart mass (mg)	151 ± 11	184 ± 37*	142 ± 22	178 ± 10*
Liver mass (g)	1.75 ± 0.16	1.62 ± 0.31	1.40 ± 0.12	1.22 ± 0.28
Quadriceps mass (mg)	171 ± 3.2	146 ± 18*	199 ± 31	135 ± 25*
Visceral fat mass (mg)	478 ± 208	335 ± 229	384 ± 162	288 ± 266
MitoQ intake (µmol/day)	n/a	n/a	1.07 ± 0.33	1.01 ± 0.28

* p<0.05 vs. YC and YMQ

Data are presented as group means ± SEM.

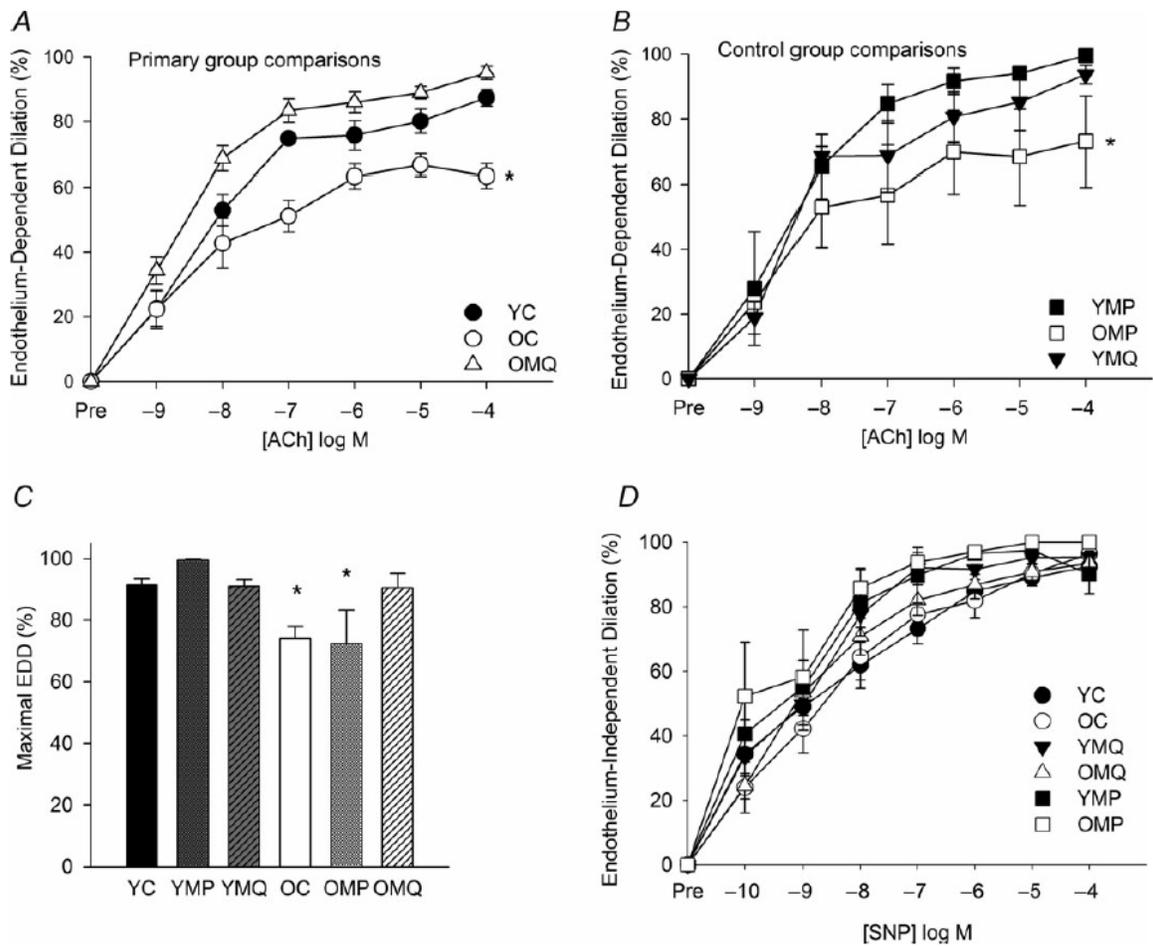


Figure 1. MitoQ reverses the age-related decline in endothelium-dependent dilation

Endothelium-dependent dilation (EDD) dose-responses to acetylcholine (ACh) in carotid arteries: A) primary group comparisons of young and old mice provided normal drinking water (YC and OC) and old mice supplemented with MitoQ (OMQ); B) control group comparisons of young and old decyl-TPP treated (YMP and OMP) and young MitoQ supplemented (YMQ) mice; * $p < 0.05$ vs. YC (main effect of group). C) Maximal EDD to acetylcholine; * $p < 0.05$ vs. YC; D) endothelium-independent dilation to the NO-donor sodium nitroprusside. Data are presented on a percentage basis to account for differences in vessel diameter among groups. Values are means \pm SEM (n=6-13/group).

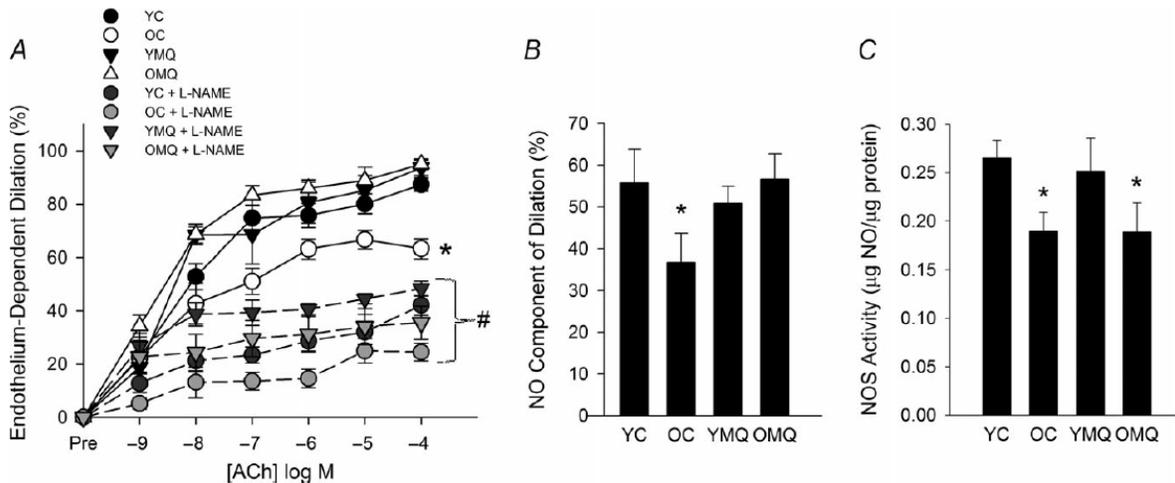


Figure 2. MitoQ restores nitric oxide-dependent endothelium-dependent dilation in old mice

A) Endothelium-dependent dilation (EDD) dose-responses to acetylcholine (ACh) in the absence/presence of the nitric oxide (NO) inhibitor N-nitro-L-arginine methyl ester (L-NAME) in carotid arteries of young and old control (YC and OC) and young and old MitoQ supplemented (YMQ and OMQ) mice. Data are presented on a percentage basis to account for differences in vessel diameter among groups. * $p < 0.05$ vs. YC (main effect of group for dose-response to ACh alone); # $p < 0.05$ within-group, dose-response to ACh + L-NAME vs. dose-response to ACh alone. There were no group differences for the dose-response with ACh+L-NAME.

B) NO-dependent dilation ($\text{maxEDD}_{\text{ACh}} - \text{maxEDD}_{\text{ACh+L-NAME}}$). * $p < 0.05$ vs. YC.

C) Total NOS activity in the aorta. * $p < 0.05$ vs. YC.

All data are presented as means \pm SEM (n=6-8/group).

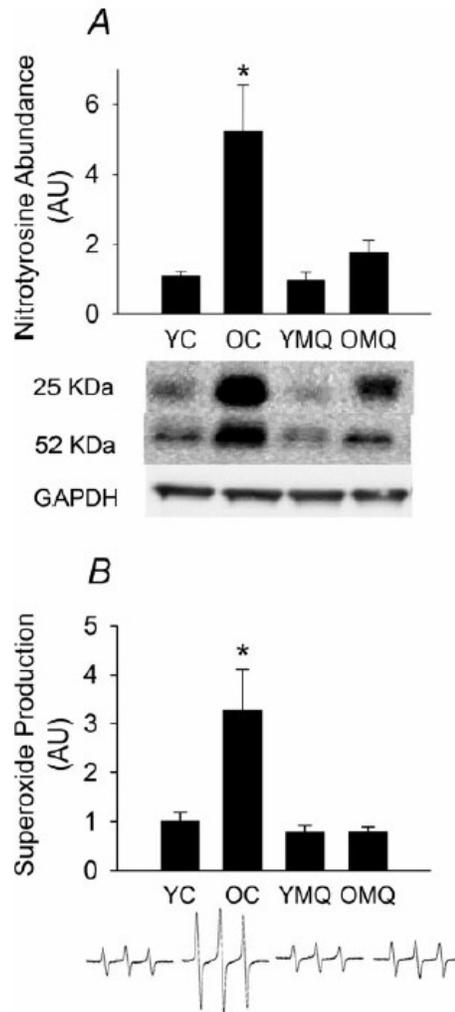


Figure 3. MitoQ normalizes vascular oxidative stress in old mice

A) Nitrotyrosine (NT), a biomarker of oxidative protein damage, in arteries (aorta) of young and old control (YC and OC) and young and old MitoQ supplemented (YMQ and OMQ) mice; representative Western blot images (25 kDa and 55kDa bands) below; B) whole-cell superoxide production in aortic segments; representative EPR spectra below. Protein expression data are normalized to GAPDH expression.

All data are normalized to YC mean values and presented as means \pm SEM (n=5-8/group).

* $p < 0.05$ vs. YC.

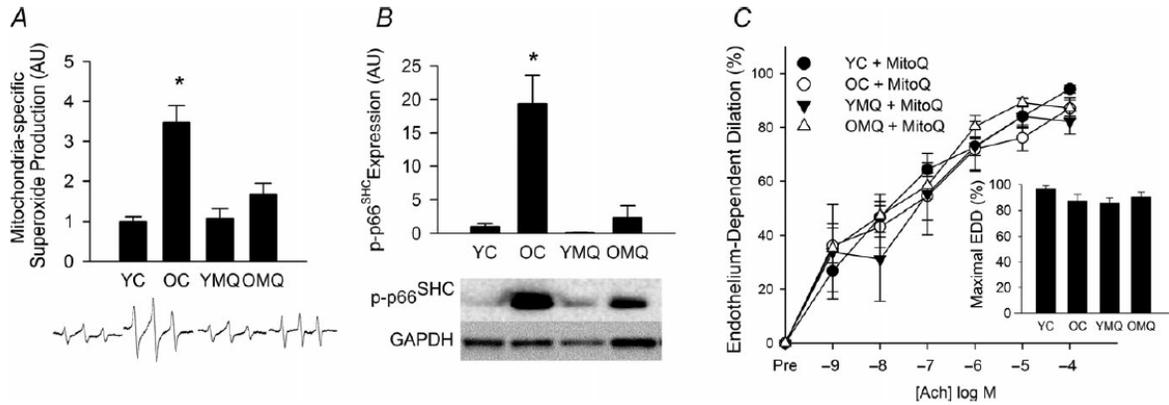


Figure 4. MitoQ reduces arterial mitochondria-derived oxidative stress and suppression of function in old mice

A) Mitochondria-specific superoxide production in aortic segments of young and old control (YC and OC) and young and old MitoQ supplemented (YMQ and OMQ) mice; representative EPR spectra below; B) aortic protein expression of phosphorylated (serine36) p66^{SHC}; representative Western blot image below; C) dose-response and maximal (inset) endothelium-dependent dilation to acetylcholine in the presence of MitoQ (1.0 μ M, 40 minute incubation to scavenge mitochondrial reactive oxygen species).

Protein expression data are normalized to GAPDH expression. Protein expression and mitochondrial superoxide data are normalized to YC mean values. All values are presented as means \pm SEM (n=6-8/group). * p<0.05 vs. YC.

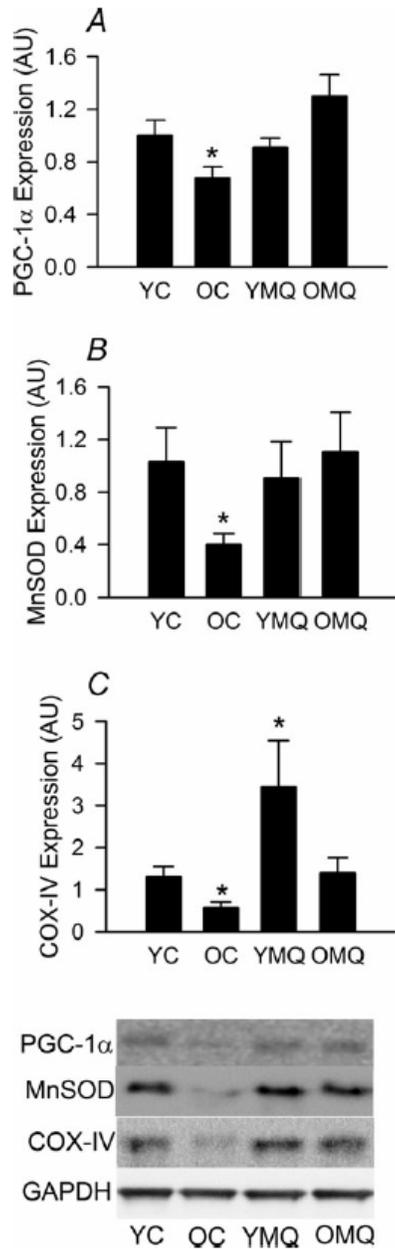


Figure 5. MitoQ improves markers of mitochondrial health in old mice

Protein expression of A) peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1 α), B) manganese superoxide dismutase (MnSOD) and C) cytochrome c oxidase (COX-IV) in aorta of young and old control (YC and OC) and young and old MitoQ supplemented (YMQ and OMQ) mice; representative Western blot images below. Protein expression data are normalized to GAPDH expression and YC mean values and presented as means \pm SEM (n=6-10/group). * p<0.05 vs. YC.

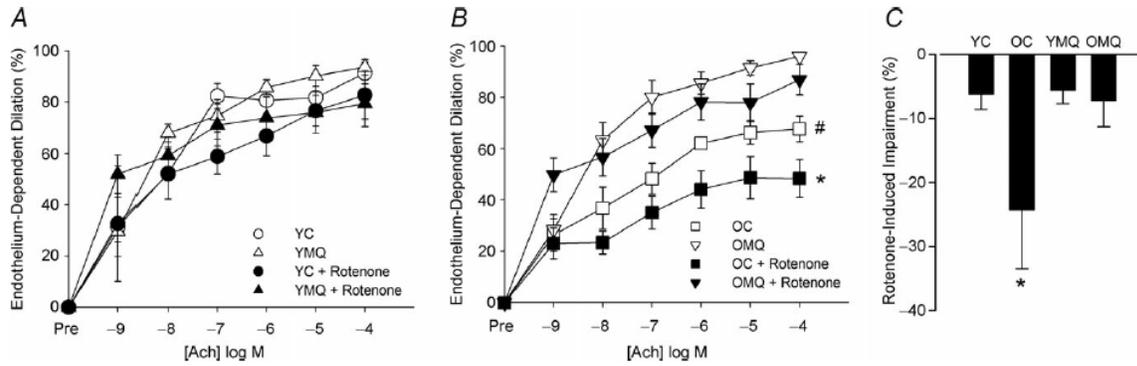


Figure 6. MitoQ improves resistance to acute mtROS stress in arteries of old mice

Endothelium-dependent dilation (EDD) dose-responses to acetylcholine (ACh) in the absence/presence of rotenone ($0.5 \mu\text{L}$, 40 minute incubation to induce mitochondrial superoxide production) in carotid arteries of A) young control and MitoQ supplemented (YC and YMQ) and B) old control and MitoQ supplemented (OC and OMQ) mice ($n=5-6/\text{group}$). Data are presented on a percentage basis to account for differences in vessel diameter among groups. Young and old data are presented separately for clarity. * $p<0.05$ within-group, dose-response to ACh + Rotenone vs. dose-response to ACh alone; # $p<0.05$ vs. OMQ (main effect of group) for dose-response to ACh alone.

C) Impairment in EDD induced by acute incubation with rotenone ($\text{maxEDD}_{\text{ACh}} - \text{maxEDD}_{\text{ACh+ROTENONE}}$). Data are presented as means \pm SEM ($n=5-6/\text{group}$). * $p<0.05$ vs. within-group maximal dilation to ACh alone.

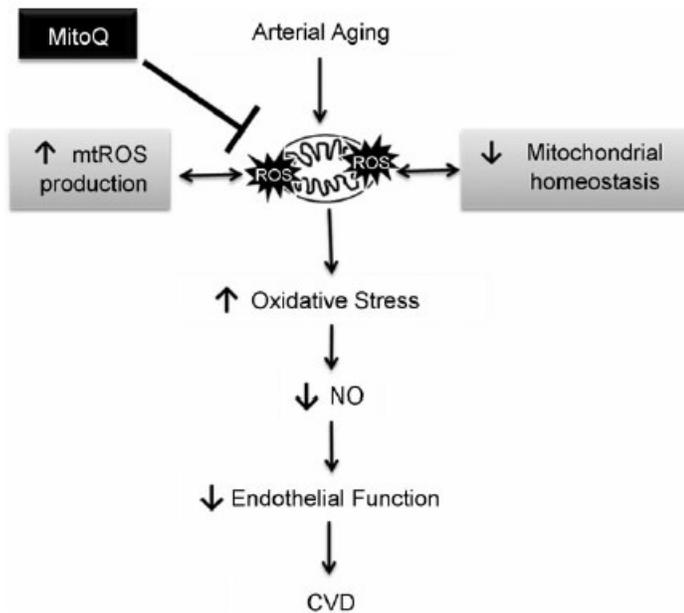


Figure 7. Working hypothesis

An increase in vascular mitochondria-derived reactive oxygen species (mtROS) production and associated dysregulation of mitochondrial homeostasis with primary aging contributes to a state of oxidative stress and a reduction in NO bioavailability, which promote the development of endothelial dysfunction. Mitochondria-targeted antioxidant treatment with MitoQ may be a promising therapeutic strategy for reducing vascular mitochondrial oxidative stress, restoring vascular mitochondrial homeostasis, and preserving endothelial function with advancing age to reduce cardiovascular disease (CVD) risk.

CHAPTER III

Mitochondria-targeted antioxidant therapy with MitoQ ameliorates large elastic artery stiffness in old mice

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ABSTRACT

Age-related arterial stiffening, a major factor underlying the age-associated increase in cardiovascular disease (CVD) risk, is mediated largely by structural changes to arteries, including increases in collagen and reductions in elastin, secondary to oxidative stress. Mitochondria are a major source of arterial oxidative stress but the potential for mitochondria-targeted therapeutic strategies to ameliorate arterial stiffness with primary aging is unknown. We assessed aortic pulse-wave velocity (aPWV), intrinsic aortic stiffness (elastic modulus of collagen and elastin regions) and aortic structural proteins in young (~ 6 mo) and old (~27 mo) male c57BL/6 mice consuming normal drinking water (YC and OC) or water containing mitochondria-targeted antioxidant MitoQ (250 μ M; YMQ and OMQ) for 4 weeks. Following the intervention period, aPWV was significantly higher in OC versus YC (482 ± 21.4 vs. 420 ± 5 cm/sec, $p < 0.05$). MitoQ treatment significantly reduced aPWV in OMQ to levels similar to young mice (426 ± 20 , $p < 0.05$ vs. OC) and had no further effect in YMQ (410 ± 10). MitoQ treatment had no effect on the age-associated increase in collagen-region elastic modulus or aortic collagen content but partially attenuated the age-associated decrease in elastin-region elastic modulus and aortic elastin content, and normalized the age-related increase in aortic matrix metalloproteinase-2 expression. Together, these results indicate that the reduction in *in vivo* arterial stiffness in old mice following MitoQ treatment was mediated by attenuation of age-related elastin degradation, and suggest that mitochondria-targeted antioxidants may represent a

novel, promising therapeutic strategy for reducing arterial stiffness with primary aging in humans.

INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of mortality in the developed world (Lloyd-Jones et al., 2010), and advancing age is a primary risk factor for the development of CVD, as greater than 90% of CVD deaths occur in adults over the age of 55 (Roger et al., 2011). The increase in CVD risk with aging is primarily attributable to the development of vascular dysfunction, including increased large elastic artery stiffness (Lakatta & Levy, 2003; Najjar et al., 2005; Mitchell et al., 2010). Indeed, aortic pulse wave velocity (aPWV), the gold-standard measure of arterial stiffness, is a strong independent risk factor for incident CV events among older adults (Mitchell et al., 2010; Sutton-Tyrell et al., 2005). Stiffening of the large elastic arteries increases the pulsatile shear and pressure experienced by the heart, blood vessels and other organs, which can have many pathophysiological effects influencing the development of CVD (Lakatta & Levy, 2003; Mitchell et al., 2010; Najjar et al., 2005; Ziemann et al., 2005). Current demographic trends predict a major increase in the number of older adults in the coming decades which will be accompanied by attendant increases in CVD prevalence and health care costs (Heidenreich et al., 2011). As such, a top biomedical research priority is to identify strategies that preserve low levels of arterial stiffness with advancing age, as this may help prevent, reduce, or delay the development of CVD.

Changes in arterial wall structure are a major mechanism by which the large elastic arteries stiffen with age (Fleenor, 2013; Greenwald, 2007; Lakatta, 2003; Ziemann et al., 2005). Specific structural changes that promote arterial stiffening include increased deposition of the load-bearing protein collagen and degradation and fragmentation of elastin, as well as increased cross-linking among structural proteins that results in further stiffening (Greenwald, 2007; Lakatta, 2003; Sell & Monnier, 2012). A key cellular mechanism underlying these structural

changes to arteries with advancing age is oxidative stress (Fleenor, 2013; Greenwald, 2007; Lakatta, 2003; Ziemann et al., 2005), a state in which the production of reactive oxygen species (ROS) exceeds the buffering capacity of endogenous antioxidant systems. Increased oxidative stress can directly and indirectly alter the activity of the enzymes involved in structural protein turnover, shifting the balance of synthesis and breakdown toward collagen deposition and elastin degradation (Fleenor, 2013; Greenwald, 2007; Lakatta, 2003; Ziemann et al., 2005).

Mitochondria are now recognized as a primary source of arterial oxidative stress with aging and cardiovascular disease (Ballinger, 2002; Dai et al., 2012; Graham et al., 2009; Vendrov et al., 2015; Zhou et al., 2011), and evidence from genetic models indicates that modulation of mtROS affects large elastic artery stiffening. For example, age-related arterial stiffness, pathological remodeling and vascular disease are accelerated in mice deficient in mitochondrial antioxidant manganese superoxide dismutase (MnSOD) (Zhou et al., 2011) and blunted in mice lacking a mitochondria-localized form of pro-oxidant enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NOX4) (Vendrov et al., 2015), suggesting that excess mtROS contribute to arterial stiffening by inducing structural changes to arteries.

Our laboratory recently demonstrated that treating old mice with the mitochondria-targeted antioxidant MitoQ to reduce mitochondrial oxidative stress completely reversed the age-related impairment in arterial endothelial function in old mice (Gioscia-Ryan et al. 2014). However, the effects of mitochondria-targeted antioxidants on aortic stiffness with primary aging have never been investigated. Therefore, in this study we tested the hypothesis that 4 weeks of MitoQ supplementation in the drinking water would reduce arterial stiffness (as assessed *in vivo* by aPWV) in old mice. To gain insights into the potential underlying mechanisms, we also

assessed the collagen- and elastin-mediated contributions to intrinsic aortic stiffness (assessed ex vivo in aortic rings) and aortic content of these key structural proteins.

METHODS

All studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder and conformed to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Mice

Male c57BL/6 mice, an established model of age-related vascular dysfunction (Fleenor et al., 2012a; Sprott & Ramirez, 1997), were purchased from the aging colony at the National Institute on Aging at ~6 or ~25 months of age and allowed to acclimate to our facilities for 2 weeks prior to beginning treatment. Mice were housed in standard cages on a 12-hour light/dark cycle and were allowed access to normal rodent chow (Harlan 7917) and water ad libitum. Body mass and water intake were monitored regularly throughout the study.

MitoQ Treatment

Based on reports of effective dose and duration of treatment with MitoQ and our previous work (Gioscia-Ryan et al., 2014; Rodriguez-Cuenca et al., 2010; Smith & Murphy, 2010), mice were randomly assigned to treatment with MitoQ (250 μ M; Antipodean Pharmaceuticals, gift from MPM) (young MitoQ-treated [YMQ, ~8 mo., n=11] and old MitoQ-treated [OMQ, ~27 mo., n=10]) or normal drinking water (young control [YC, ~8 mo., n=8] and old control [OC, ~27 mo., n=10]) for 4 weeks, a duration we have previously shown to be effective in reversing age-related arterial endothelial dysfunction (Gioscia-Ryan et al., 2014). MitoQ was prepared fresh and administered in light-protected water bottles changed every three days.

In Vivo Assessment of Arterial Stiffness: Aortic Pulse-Wave Velocity

In vivo arterial stiffness was assessed at baseline and after 4 weeks of MitoQ treatment by aortic pulse-wave velocity (aPWV) using Doppler ultrasound, as previously described by our laboratory (Fleenor et al., 2012a (nitrite); LaRocca et al., 2014). Briefly, mice were anesthetized via inhaled isoflurane (1.5-2%) and positioned supine on a warmed platform with paws secured to ECG leads. Doppler probes were placed at the transverse aortic arch and abdominal aorta to detect pulse waves. Three consecutive 2-second recordings were made for each animal and used to determine time delay between the ECG R-wave and the foot of the Doppler signal for each site ($\Delta\text{time}_{\text{abdominal}}$ and $\Delta\text{time}_{\text{transverse}}$). aPWV was then calculated as $\text{aPWV} = (\text{physical distance between the two probes}) / (\Delta\text{time}_{\text{abdominal}} - \Delta\text{time}_{\text{transverse}})$.

To examine the potential role of changes in blood pressure to treatment-related differences in aPWV, we assessed systolic and diastolic blood pressure at baseline and following 4 weeks of MitoQ or normal drinking water consumption using the CODA non-invasive tail-cuff system as previously described (Fleenor et al., 2014; LaRocca et al., 2014). The pressure measurements from 20 collection cycles (following 5 acclimation cycles) on each of three consecutive days were averaged for each mouse at each timepoint.

Ex-vivo Assessment of Arterial Stiffness: Intrinsic Mechanical Stiffness

Following all *in vivo* assessments, mice were euthanized and aortas were harvested for measurements of *ex-vivo* intrinsic mechanical stiffness and protein expression. Two 1-mm aortic rings (dissected free of surrounding connective tissue) were used to assess intrinsic aortic stiffness via wire myography, as described previously by our laboratory (de Picciotto et al., 2016; Fleenor et al., 2012a; Fleenor et al., 2014; LaRocca et al., 2014). Aortic rings were loaded into heated myograph chambers (DMT, Inc.) with calcium-free phosphate buffered saline.

Following three cycles of pre-stretching, ring diameter was increased to achieve 1mN force and then incrementally stretched by 10% every 3 minutes until failure. The force corresponding to each stretching interval was recorded and used to calculate stress and strain, defined as follows:

$$\text{Strain } (\lambda) = \Delta d/d(i)$$

d= diameter; d(i)= initial diameter

$$\text{Stress } (t) = \lambda L/2HD$$

L= one-dimensional load; H= wall thickness determined by histology; D= vessel length

The slope of the stress-strain curve was used to determine the elastic modulus in the collagen-dominant and elastin-dominant regions of the curve, as described below.

Collagen elastic modulus

When aortic rings are subjected to stress-strain testing, the region of the stress-strain curve corresponding to the highest forces represents the stretching of predominately collagen fibers (Sokolis et al., 2002; Lammers et al., 2008). The elastic modulus of the collagen-dominant region was determined as the slope of the linear regression fit to the final four points of the stress-strain curve, as described previously (de Picciotto et al., 2016; Fleenor et al., 2014), LaRocca et al., 2014).

Elastin elastic modulus

During stress-strain testing in aortic rings, the region of the stress-strain curve corresponding to the stretching of exclusively elastin fibers is a lower-force region prior to collagen fiber engagement that can be identified as the portion of the stress-strain curve where curvature (determined from the second derivative of the stress-strain curve) is approximately zero; the engagement of collagen fibers is indicated by an elevation in the curvature (non-zero second derivative) (Lammers et al., 2008). To determine the boundaries of the elastin region of

our stress strain curves, we calculated the roots of the second derivative of a 7th order polynomial fit to the data ($R^2 > 0.99$). The first root was considered the boundary between the very low-force region and the elastin region, and the second root was considered the boundary between the elastin region and the onset of collagen fiber engagement (Lammers et al., 2008). The elastic modulus of the elastin region was then determined as the slope of the linear regression fit to the stress-strain data between the two points.

Aortic Protein Expression

Protein expression was determined in aortic homogenates by standard Western blotting techniques and immunohistochemistry (IHC) in aortic sections, as previously described (de Picciotto et al., 2016; Fleenor et al., 2010; LaRocca et al., 2014). For Western blotting, aortas were homogenized in radio-immunoprecipitation assay lysis buffer. Following homogenization, 15 μ g of aortic protein were loaded onto 4-12% polyacrylamide gels and then transferred onto nitrocellulose membranes (Criterion System; Bio-Rad, Hercules, CA, USA). Membranes were incubated (overnight at 4°C) with primary antibodies: collagen-I (1:1000, Millipore Corp.), α -elastin (1:200, Abcam, Inc., Cambridge, MA, USA), matrix metalloproteinase-2 (MMP-2; 1:200, Santa Cruz), lysyl oxidase (LOX; 1:500, Novus Biologicals) and glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling, 1:1000, normalizer). Proteins were visualized on a digital acquisition system (ChemiDoc-It, UVP, Upland, CA, USA) using chemiluminescence with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Westgrove, PA, USA) and ECL substrate (Pierce, Rockford, IL, USA). Relative intensity was quantified using ImageJ software and normalized to GAPDH intensity and then expressed as a ratio of the mean intensity of the young control group.

For IHC, aortic segments were frozen in OCT compound in liquid nitrogen-cooled isopentane. Aortic sections (7 μ m) were fixed in acetone, washed in Tris buffer, and stained using the Dako EnVision+ System-HRP-DAB kit, as performed previously in our laboratory (Fleenor et al., 2010). Sections were incubated for 1 h at 4°C with primary antibodies for α -elastin (1:50, Abcam Inc.) and collagen-I (1:200, Millipore) and then incubated with the labelled polymer secondary for 30 minutes. Staining was visualized after a 10-minute or 1-minute exposure to diaminobenzidine (elastin and collagen, respectively) and then slides were dehydrated and coverslipped.

Stained aortic sections were imaged using a Nikon Eclipse TS100 photomicroscope under identical conditions for all sections for each protein. Quantification of the integrated density of the stain was performed using ImageJ software for the entire region of interest by a single investigator blinded to the group assignment of each sample. Integrated density values from 4 sections of each aorta were averaged to provide a single value for each protein.

Statistical Analysis

All statistical analyses were performed using SPSS 23.0 software (Armonk, NY, USA). Data were assessed for outliers and normality/homogeneity of variance. When assumptions of normality were met, between-group differences in elastic modulus and aortic protein expression were determined using a linear mixed model with age (young versus old) and treatment (control versus MitoQ) as factors, whereas within-group differences in aPWV were examined using a linear mixed model that included a repeated factor (pre versus post intervention period). When a significant main effect was observed, Fisher's least significant difference post-hoc tests were performed to determine specific pair-wise differences.

RESULTS

MitoQ consumption across the 4-week treatment period was similar to our previous report and not different between young and old mice (~1 mmol/day; Gioscia-Ryan et al., 2014). Select morphological characteristics and blood pressure are shown in **Table 1**. Consistent with our previous study (Gioscia-Ryan et al., 2014), MitoQ treatment did not influence overall morphology; although there were age-associated differences in body mass, heart mass, and quadriceps mass, these were not different between mice receiving MitoQ versus normal drinking water. There were no age- or treatment-related differences in aortic diameter or systolic and diastolic blood pressure.

MitoQ treatment reduces arterial stiffness in old mice

At baseline, aPWV was significantly higher in old compared to young mice and aPWV was not significantly different from baseline to post-intervention in either young or old control mice receiving normal drinking water (**Figure 1**). In contrast, 4 weeks of MitoQ treatment significantly reduced aPWV in old mice to levels similar to young mice following the intervention period. MitoQ treatment had no significant effect on aPWV in young mice. These results indicate that 4 weeks of MitoQ treatment in old mice reverses age-associated large elastic artery stiffening.

MitoQ treatment attenuates the age-related decline in elastin-mediated mechanical properties but has no effect on collagen-mediated mechanical stiffness

The elastic modulus of the collagen region of stress-strain curves was significantly greater in old control versus young control mice (**Figure 2A**), whereas the elastic modulus of the elastin region was significantly lower in old control compared to young control mice (**Figure 2B**), indicating an age-related increase in intrinsic arterial stiffness mediated by increased arterial

collagen and reduced arterial elastin content. MitoQ treatment had no effect on the collagen elastic modulus, such that the values in old and young MitoQ-treated mice were not significantly different from old and young control mice, respectively. However, in arteries from old mice treated with MitoQ, the elastic modulus in the elastin region was significantly greater than that of old control mice but remained significantly lower than the elastin elastic modulus of young MitoQ-treated mice, indicating partial attenuation of the age-related decline in elastin.

MitoQ treatment attenuates the age-related decline in aortic elastin content

Consistent with our intrinsic mechanical stiffness observations, aortic collagen protein expression was greater (**Figure 3A and B**) and aortic elastin expression was lower (**Figure 3C and D**) in old control versus young control mice. MitoQ treatment did not alter aortic collagen content, such that collagen levels in old MitoQ-treated mice were not significantly different than those of old control mice, whether assessed in whole artery homogenate by Western blot or in aortic sections via IHC. When measured in whole artery homogenate by Western blot, aortic elastin content in old MitoQ-treated mice was not significantly different from that of either young control or old control mice. However, when assessed via IHC in the medial layer of aortas—the primary site of age-related elastin degradation (Fleenor et al., 2010; Fleenor, 2013)—elastin content in old MitoQ-treated mice was greater than that of old control mice ($p=0.07$).

Together, these results suggest that the reduction in *in vivo* arterial stiffness in old mice following MitoQ treatment was mediated not by effects on aortic collagen content, but rather by attenuation of the age-related decline in elastin content.

MitoQ treatment normalizes aortic MMP-2 expression in old mice but does not alter LOX expression

Expression of the key elastin-degrading enzyme MMP-2 was significantly higher in old control compared to young control mice (**Figure 4A**). MitoQ treatment reduced aortic MMP-2 expression in old mice and had no further effect in young mice. We did not observe any significant differences in aortic expression of elastin-synthesizing enzyme LOX with age or MitoQ treatment (**Figure 4B**). These results suggest that the preservation of elastin content with MitoQ treatment may be due to a reduction in MMP-2-mediated elastin degradation.

DISCUSSION

The primary, novel finding of this study is that 4 weeks of treatment with the mitochondria-targeted antioxidant MitoQ in old mice completely reverses the age-associated increase in arterial stiffness, assessed *in vivo* as aPWV, and this is mediated at least partially by attenuation of the age-related reduction in arterial elastin content.

Our observation that MitoQ treatment reduces large elastic artery stiffness in old mice extends previous work with general antioxidant compounds and adds to the evidence from transgenic/disease models that specifically implicates *mitochondrial* oxidative stress as a key contributor to arterial stiffness. A previous pre-clinical intervention study employing the general antioxidant compound TEMPOL established oxidative stress as a key mechanism underlying age-related arterial stiffening (Fleenor et al., 2012b), and other strategies that reduce arterial oxidative stress also ameliorate arterial stiffness (Fleenor et al., 2010; Fleenor et al., 2012a; Fleenor et al., 2012c; LaRocca et al., 2014; Stepan et al., 2012). Recent work with genetic mouse models now indicates that mitochondria are a major source of the vascular oxidative stress contributing to arterial stiffness. Mice with genetic deletion of mitochondrial antioxidant

enzyme MnSOD, a model of excess mitochondrial oxidative stress, demonstrate exacerbation of age-related arterial stiffening (Zhou et al., 2011), whereas mice with genetic deletion of mitochondria-localized pro-oxidant enzyme NOX4, a model of reduced mitochondrial oxidative stress, demonstrate lower arterial stiffness compared to wild-type controls (Vendrov et al., 2015). Our finding here that treatment with the mitochondria-targeted antioxidant MitoQ treatment in old mice reduces arterial stiffness provides further support for mitochondrial oxidative stress as a key mediator of arterial dysfunction with primary aging.

To gain initial mechanistic insight into the de-stiffening effects of MitoQ treatment, we assessed intrinsic mechanical stiffness *ex-vivo* in aortic rings and examined both the collagen- and elastin-predominant regions of the stress-strain curves. In contrast to previous studies showing that the de-stiffening effects of late-life interventions, including those associated with reduced oxidative stress, are primarily mediated by reductions in arterial collagen content (Fleenor et al., 2010; Fleenor et al., 2012b; Fleenor et al., 2012c; Fleenor, 2013; Nosaka et al., 2003), we observed that MitoQ treatment had no significant effect on the collagen region elastic modulus or aortic collagen content but instead attenuated age-related declines in both aortic elastin region elastic modulus and elastin content. Our finding of elastin preservation with MitoQ treatment is consistent with the observations that heterozygous MnSOD deficient mice, a model of excess mtROS, show marked exacerbation of age-associated declines in arterial elastin content (Zhou et al., 2011), and that lifelong caloric restriction, a setting of reduced mtROS (Lanza et al., 2012), also preserves arterial elastin content with aging (Donato et al., 2012).

Our finding that MitoQ treatment attenuated the age-associated reduction in aortic elastin is also supported by evidence that mtROS are critical regulators of enzymes involved in elastin turnover, including the matrix metalloproteinases (MMPS) (Nelson & Melendez, 2004). In

heterozygous MnSOD knockout mice, the loss of arterial elastin is accompanied by an increase in MMP-2 (Zhou et al., 2011) a key enzyme involved in elastin degradation (Fleenor, 2013; Greenwald, 2007; Wang & Lakatta, 2002). Primary aging in preclinical models is associated with increased arterial MMP-2 expression (Fleenor et al., 2010; Wang & Lakatta, 2002), and elevated aortic MMP-2 levels are also observed in human aging (McNulty et al., 2005). We observed that aortic MMP-2 expression was reduced in old mice following MitoQ treatment, whereas expression of LOX, an elastin-synthesizing enzyme, was unaltered by MitoQ treatment. Collectively, our results suggest that targeting mitochondria may attenuate elastin degradation, preserving elastin content in large elastic arteries and contributing to lower levels of stiffness.

Limitations and future directions

It is also important to consider mechanisms other than preservation of aortic elastin content that may have contributed to the dramatic reduction in arterial stiffness we observed with MitoQ treatment in old mice. In addition to structural changes, age-related arterial stiffening is also mediated by hemodynamic factors (including age-related reductions in vascular endothelial function) and increased vasomotor tone (Greenwald, 2007; Lakatta, 2003; Zieman et al., 2005). Although our data indicate that changes in resting blood pressure did not contribute to the effects of MitoQ treatment, it is plausible that some of the de-stiffening we observed in old mice was due to improvements in vascular endothelial function. Our previous study (Gioscia-Ryan et al., 2014) demonstrated that MitoQ treatment increases endothelium-dependent dilation and nitric oxide bioavailability in old mice, both of which are important direct (e.g., effects on pulse pressure and smooth muscle tone) and indirect (e.g., regulation of structural protein turnover) mediators of large elastic artery stiffness *in vivo* (Greenwald, 2007; Wilkinson et al., 2004; Zieman et al., 2005).

Although our results do not support a role for MitoQ in reducing total arterial collagen content, future studies could examine not only arterial content of this key structural protein, but also changes in collagen fiber orientation (Greenwald, 2007) and formation of cross-links among proteins, both of which have the potential to influence arterial stiffness (Fleenor, 2013; Lakatta & Levy, 2003; Ziemann et al., 2005). Additionally, the regulation of structural protein turnover is complex and involves many more enzymes, including other members of the MMP family and their inhibitors, than were investigated here (Greenwald, 2007; Liu et al., 2015; Ziemann et al., 2005). Because the activity of many of these enzymes is influenced by redox status (Nelson & Melendez, 2004; Sternlicht & Werb, 2001), future work could investigate how reducing mitochondrial oxidative stress with MitoQ influences activity of these enzymes, and the effects on structural protein turnover.

In conclusion, the present study demonstrates for the first time that late-life treatment with a mitochondria-targeted antioxidant, MitoQ, effectively reverses age-related large elastic artery stiffening, and that this effect is mediated at least partially by attenuation of the age-related reduction in arterial elastin content. These results suggest that mitochondria-targeted antioxidants may represent a novel, promising therapeutic strategy for reducing arterial stiffness, and reducing CVD risk, with primary aging in humans.

ACKNOWLEDGEMENTS

The authors thank Dr. Blair Dodson and Jesse Goodrich for study assistance.

FUNDING

This study was supported by NIH grants AG047784 (RGR); AG000279 (RGR); HL107120-04 (DRS) and AG0138038 (DRS)

Table 1. General morphological characteristics and blood pressure

	YC	OC	YMQ	OMQ
Body mass (g)	25.1 ± 1.2	29.4 ± 2.7*	26.0 ± 1.4	28.5 ± 3.0*
Heart mass (mg)	128 ± 11	175 ± 22*	124 ± 9	164 ± 20*
Liver mass (g)	1.34 ± 0.06	1.41 ± 0.16	1.34 ± 0.18	1.37 ± 0.38
Quadriceps mass (mg)	163 ± 2.8	138 ± 27*	175 ± 29	143 ± 27*
Visceral fat mass (mg)	306 ± 70	302 ± 89	256 ± 71	229 ± 118
Aorta diameter (µm)	749 ± 72	780 ± 47	789 ± 77	785 ± 43
Systolic BP (mmHg)	Pre: 105.1 ± 10.1	Pre: 101.3 ± 11.7	Pre: 101.2 ± 6.7	Pre: 93.5 ± 10.8
	Post: 101.4 ± 12.9	Post: 94.9 ± 5.0	Post: 98.2 ± 10.8	Post: 101.0 ± 4.4
Diastolic BP (mmHg)	Pre: 73.3 ± 11.5	Pre: 72.8 ± 11.0	Pre: 73.1 ± 4.7	Pre: 66.3 ± 4.2
	Post: 71.9 ± 11.3	Post: 67.0 ± 4.6	Post: 74.1 ± 9.3	Post: 71.9 ± 10.1

Data are presented as means ± SD. YC, young control mice; OC, old control mice; YMQ, young MitoQ-treated mice; OMQ, old MitoQ-treated mice; BP, blood pressure; Pre, baseline assessment (prior to treatment period); Post, assessment following 4-week treatment period with MitoQ or normal drinking water).

* p<0.05 vs. YC and YMQ

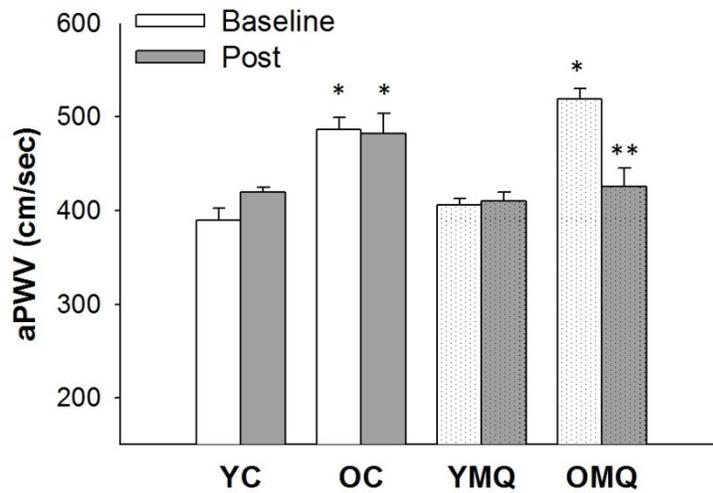


Figure 1. MitoQ treatment reverses age-related arterial stiffness in mice

Aortic pulse-wave velocity (aPWV) was assessed in young and old mice before (baseline) and following (post) consumption of normal drinking water (YC and OC) or MitoQ treatment (YMQ and OMQ) for 4 weeks. n=8-11/group; error bars represent SEM.

* p<0.05 vs. YC and YMQ; ** p<0.05 vs. OC and OMQ baseline

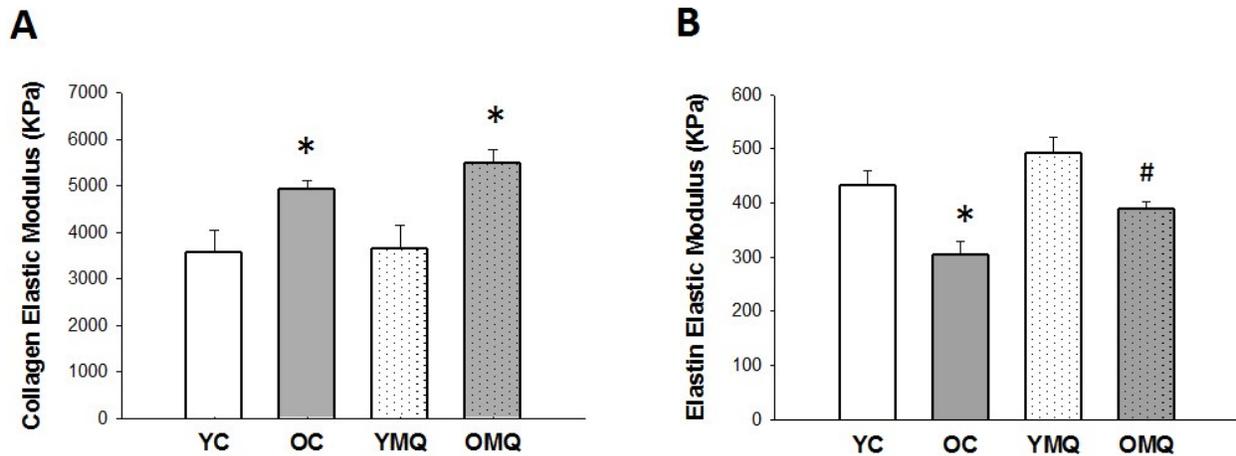


Figure 2. MitoQ treatment attenuates the age-related decline in elastin-mediated intrinsic mechanical properties but has no effect on collagen-mediated intrinsic mechanical stiffness

A: Collagen region elastic modulus of aortic segments from young and old control (YC and OC) and young and old MitoQ-treated (YMQ and OMQ) mice.

B: Elastin region elastic modulus of aortic segments from YC, OC, YMQ and OMQ mice. n=8-11/group; error bars represent SEM.

* $p < 0.05$ vs. YC and YMQ

$p < 0.05$ vs. OC and YMQ

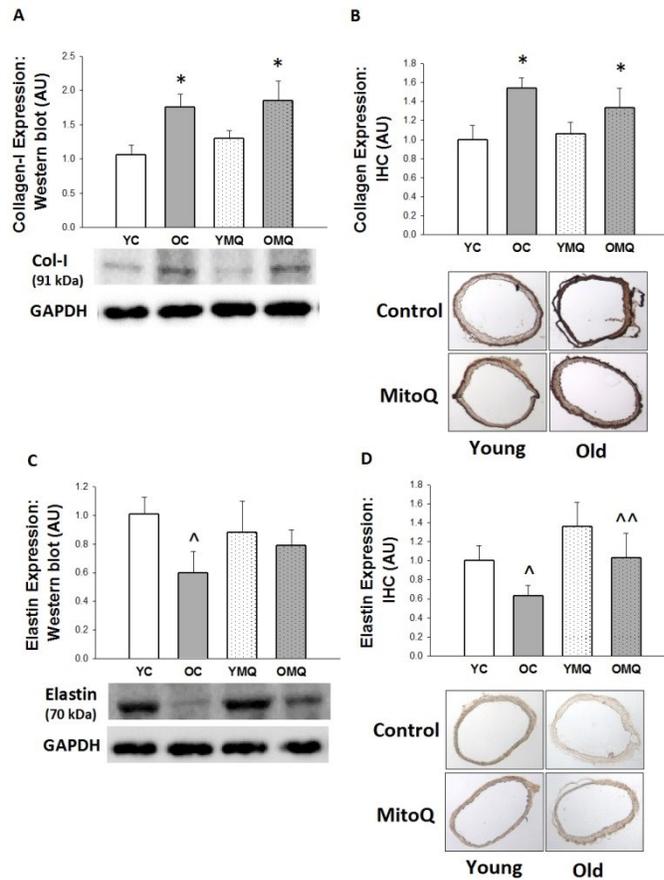


Figure 3. MitoQ treatment attenuates the age-related reduction in aortic elastin expression

A: Aortic collagen expression assessed by Western blot in aortic homogenates from young and old control (YC and OC) and young and old MitoQ-treated (YMQ and OMQ) mice. Expression levels are presented normalized to GAPDH expression and relative to the mean of the YC group (error bars represent SEM). Representative images are presented below mean data. n=6/group
* p<0.05 vs. YC and YMQ

B: Aortic collagen expression assessed by immunohistochemistry in aortic sections from YC, OC, YMQ and OMQ mice. Expression levels are presented relative to the mean of the YC group (error bars represent SEM). Representative images are presented below mean data. n=4/group
* p<0.05 vs. YC and YMQ

C: Aortic elastin expression assessed by Western blot in aortic homogenates from young and old control (YC and OC) and young and old MitoQ-treated (YMQ and OMQ) mice. Expression levels are presented normalized to GAPDH expression and relative to the mean of the YC group (error bars represent SEM). Representative images are presented below mean data. n=6/group
[^] p<0.074 vs. YC

D: Aortic elastin expression assessed by immunohistochemistry in aortic sections from YC, OC, YMQ and OMQ mice. Expression levels are presented relative to the mean of the YC group (error bars represent SEM). Representative images are presented below mean data. n=8-11/group
[^] p=0.086 vs. YC; ^{^^} p=0.075 vs. OC

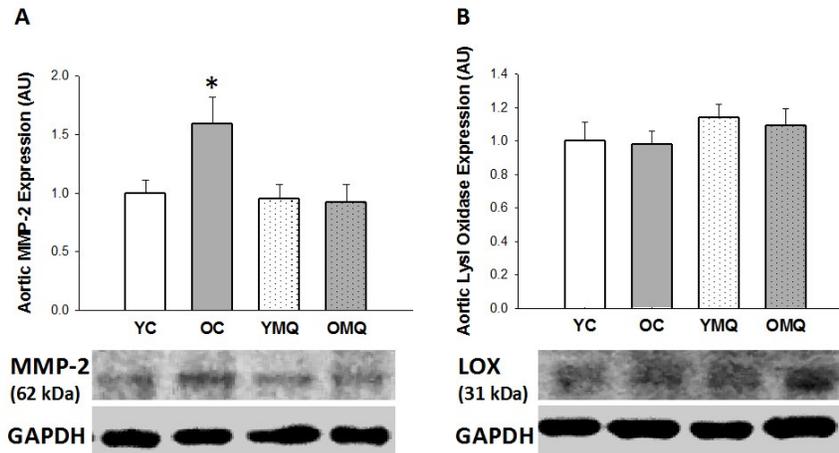


Figure 4. MitoQ treatment in old mice reduces aortic MMP-2 expression

Aortic expression of matrix metalloproteinase-2 (MMP-2) and lysyl oxidase (LOX) in aortic homogenates from young and old control (YC and OC) and young and old MitoQ-treated (YMQ and OMQ) mice. Expression levels are presented normalized to GAPDH expression and relative to the mean of the YC group (error bars represent SEM). Representative images are presented below mean data. n=4-6/group

* $p < 0.05$ vs. all other groups

CHAPTER IV

Voluntary aerobic exercise increases arterial resilience and mitochondrial health with aging in mice

Gioscia-Ryan RA, Battson ML, Cuevas LM, Zigler MC, Sindler AL, Seals DR.

ABSTRACT

Mitochondrial dysregulation and associated excessive reactive oxygen species (mtROS) production is a key source of oxidative stress in aging arteries that reduces baseline arterial function and may also influence arterial resilience (ability to withstand stress). Aerobic exercise restores baseline endothelial function with aging and improves mitochondrial health in many tissues. We hypothesized that voluntary aerobic exercise would increase arterial resilience to acute stressors in old mice, accompanied by improved arterial mitochondrial health. Ten weeks of voluntary wheel running (VWR) reversed the age-associated decline in endothelium-dependent dilation (EDD) and normalized basal arterial mtROS levels. An acute mitochondrial stressor (rotenone) caused greater (further) impairment in peak EDD in old (OC; $-32.5 \pm -10.5\%$) versus young (YC; $-5.4 \pm -3.7\%$) control mice, whereas arteries from young and old exercising (YVR and OVR; $-0.8 \pm -2.1\%$ and $-8.0 \pm 4.9\%$, respectively) mice were protected. Ex-vivo treatment with simulated Western diet (WD) caused greater impairment in EDD in OC ($-28.5 \pm 8.6\%$) versus YC ($-16.9 \pm 5.2\%$) and YVR ($-15.3 \pm 2.3\%$), whereas OVR ($-8.9 \pm 3.9\%$) were more resilient (not different versus YC). Simultaneous ex-vivo treatment with the mitochondria-specific antioxidant MitoQ selectively attenuated WD-induced impairments in YC and OC, but not YVR or OVR, suggesting that exercise improved resilience to mtROS-mediated stress. Exercise also normalized age-related alterations in protein markers of arterial mitochondrial health and augmented cellular antioxidant and stress response proteins. Our results indicate that

arterial aging is accompanied by reduced resilience and mitochondrial health, which are restored by voluntary aerobic exercise.

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death in developed societies (Lloyd-Jones et al. 2010). The risk of CVD increases progressively with advancing age, such that greater than 90% of deaths from CVD occur in people over the age of 55 (Roger et al. 2012). Although the mechanisms underlying the age-related increase in CVD risk have not been fully elucidated, strong evidence indicates that the development of arterial dysfunction is a key factor (Herrera et al. 2010; Yeboah et al. 2007). An important manifestation of arterial dysfunction is vascular endothelial dysfunction, characterized by a decline in endothelium-dependent dilation (EDD) (Bachschmid et al. 2013; Lakatta 2003; Seals et al. 2014).

One of the major mechanisms underlying the development of age-related endothelial dysfunction is oxidative stress, a state in which production of reactive oxygen species (ROS) exceeds the capacity of endogenous antioxidant defense systems. In the aging vasculature, oxidative stress reduces bioavailability of the vasodilatory and vasoprotective molecule nitric oxide (NO), leading to declines in EDD (Brandes et al. 2005; van der Loo et al. 2000; Lakatta 2003; Seals et al. 2014; Bachschmid et al. 2013).

A key source of arterial oxidative stress is excessive production of mitochondrial reactive oxygen species (mtROS). Healthy mitochondria are critical mediators of arterial homeostasis (Kluge et al. 2013; Dai et al. 2012; Dromparis & Michelakis 2013; Gioscia-Ryan et al. 2014, LaRocca et al., 2014) and produce physiological levels of mtROS vital for cell signaling (Widlansky & Gutterman 2011). However, declines in mitochondrial health are characterized by excessive mtROS production (Quintero et al. 2006; Dromparis & Michelakis 2013; Dai et al. 2012, Kluge et al. 2013; Widlansky & Gutterman 2011). We have recently shown that excess arterial mtROS production is a major contributor to tonic arterial oxidative stress-mediated

suppression of EDD with primary aging in mice (Gioscia-Ryan et al. 2014), as scavenging of mtROS with a mitochondria-specific antioxidant completely restores EDD in old mice.

Emerging evidence suggests that, in addition to baseline deficits in vascular function, aging may also be accompanied by reduced arterial resilience, i.e., the ability to withstand stress. Aging exacerbates the effects of common in vivo stressors such as a “Western”-style (high fat/high sugar) diet, hyperglycemia, and elevated low-density lipoprotein (LDL) cholesterol, such that the age- and stressor-associated impairments are compounded, resulting in a greater degree of impairment (Lesniewski et al. 2013; Walker et al. 2009; DeVan et al. 2013; Seals et al. 2014; Ungvari et al. 2010). Because human aging occurs in the presence of numerous stressors, it is important to understand how aging alters arterial resilience and to identify potential interventions that may improve the ability of arteries to withstand these challenges.

Mitochondria are critical components of the cellular stress response and interact with and regulate other stress response mediators, including antioxidant enzymes and heat shock proteins (Hsp) (Bause & Hagsis 2013; Bell & Guarente 2011; Gielen et al. 2010; Manoli et al. 2007; Noble et al. 2008). Thus, mitochondrial dysregulation has the potential to impact major upstream mechanisms, such as oxidative stress, that mediate vascular function (Marzetti et al. 2013). Indeed, recent work indicates that arterial functional impairments are linked to declines in arterial mitochondrial health and that restoration of arterial function is accompanied by concomitant improvements in arterial mitochondrial health, underscoring the importance of healthy mitochondria for arterial function (Gioscia-Ryan et al. 2014; LaRocca et al. 2014; Keller et al. 2015; Knaub et al. 2013; Marzetti et al. 2013; Young et al. 2004). However, it is unknown whether age-related declines in arterial mitochondrial health contribute to decreased resilience in the presence of acute stressors.

Aerobic exercise is a powerful intervention that improves baseline endothelial function in the setting of aging (Seals 2014, Seals et al. 2009; Seals et al. 2008, Lesniewski et al. 2013; Durrant et al. 2009). It is well known that aerobic exercise improves mitochondrial biogenesis and homeostasis in non-vascular tissues (Koltai et al. 2012; Hood et al. 2011; Meshnikova et al. 2006; Irrcher et al. 2003; Ljubcic et al. 2010; Handschin 2009), and recent work suggests that exercise can also improve markers of arterial mitochondrial content and health in healthy animals (Gu et al. 2014; Keller et al. 2015; Knaub et al. 2013; Miller et al. 2013; Park et al. 2016). However, these studies also indicate that the vascular mitochondrial response to exercise is impaired in certain disease models and settings, including hypertension, diabetes and NO deficiency (Gu et al. 2014; Keller et al. 2015; Knaub et al. 2013; Miller et al. 2013; Young et al. 2004), but the effects of aerobic exercise on arterial mitochondria with primary aging are unclear. We tested the hypothesis that aging would be associated with impaired arterial resilience to acute stress and reduced arterial mitochondrial health, and that voluntary aerobic exercise initiated in late-life would increase resilience and improve mitochondrial health in aging arteries.

METHODS

Ethical approval

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder and conformed to the standards published in the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

Animals and exercise intervention

Male c57Bl/6 mice were obtained from the colony at the National Institute on Aging (Bethesda, Maryland, U.S.) at ~5 or ~25 months of age. Animals were allowed to acclimate to our facility for 2 weeks, were kept on a 12-hour light:dark cycle and were provided normal

rodent chow (Harlan 7019) and water ad libitum. Following the 2-week acclimation period, mice were randomly assigned to either a sedentary cage control group (young control [YC], n=11 and old control [OC], n=12) or to a voluntary aerobic exercise group (young voluntary wheel running [YVR], n=10 and old voluntary wheel running [OVR], n=11) for 10 weeks. The animals in the voluntary aerobic exercise groups were housed in cages with access to a running wheel (Lafayette Instruments, Lafayette, IN, USA) and were permitted to exercise ad libitum. Daily wheel running was monitored using Activity Wheel Monitor software (Lafayette, IN, USA) for 72 continuous hours once per week, and daily running activity was determined as the average distance run per 24 hour period. Following the 10-week intervention period, animals were killed by exsanguination via cardiac puncture under inhaled isoflurane anesthetic, and tissues and organs were harvested for assessment of arterial function and biochemical parameters as described below.

Vascular endothelial function

Vascular endothelial function was assessed in isolated carotid arteries (2 per animal), as described previously (Gioscia-Ryan et al. 2014; LaRocca et al. 2012). Briefly, arteries were dissected and cleared of surrounding tissue and cannulated onto glass micropipettes in a myograph chamber containing warmed (37 °C) physiological saline solution. Arteries were pressurized to ~ 50mm Hg and allowed to equilibrate for 45 minutes. Following equilibration, the arteries were pre-constricted with 2 μ M phenylephrine (Sigma Aldrich Corp., St. Louis, MO, USA), and endothelium-dependent dilation was assessed as the magnitude of dilation in response to increasing doses of acetylcholine (ACh, 1×10^{-9} – 1×10^{-4} M, Sigma Aldrich Corp.). Following this assessment of baseline endothelial function, we next assessed endothelium-dependent dilation in the presence of acute stressors (as described below) in the same arterial segments.

Following all measurements of endothelium-dependent dilation, endothelium-independent dilation was assessed as the magnitude of dilation in response to increasing doses of sodium nitroprusside (SNP, 1×10^{-10} - 1×10^{-4} M, Sigma Aldrich Corp.), an exogenous NO donor. All vessel data are presented on a percentage basis to account for baseline differences in vessel diameter among animals. Peak EDD (greatest value of endothelium-dependent dilation) and the area under the dose-response curve (AUC, trapezoid method) were determined for each response.

Acute mitochondria-specific stress

To determine the effects of an acute mitochondria-specific stress on endothelial function, following assessment of dilation in response to ACh alone as described above, a sub-set of arteries (n = 7 (YC), 8 (OC), 5 (YVR), 6 (OVR)) was incubated with 0.5 μ M rotenone (Sigma Aldrich Corp.), a mitochondrial respiratory Complex I inhibitor, for 40 minutes prior to assessment of EDD to ACh, as previously described (Gioscia-Ryan et al. 2014). This low dose of rotenone has been shown to increase mitochondrial superoxide production from Complex I without completely inhibiting respiratory activity (Li et al. 2003; Weir et al. 1991). The rotenone-induced impairment in peak EDD was determined as the relative reduction in peak dilation in the presence versus absence of rotenone ($[\text{PeakEDDACh} - \text{PeakEDDRotenone} / \text{PeakEDDACh}] \times 100$). Similarly, the rotenone-induced impairment in the EDD AUC was determined as the relative reduction in the AUC in the presence versus absence of rotenone ($[\text{AUCACh} - \text{AUCRotenone} / \text{AUCACh}] \times 100$).

Acute ex-vivo simulated Western diet stress

To determine the effects of acute exposure to a more physiologically relevant stressor, we exposed a sub-set of arteries (n = 7 (YC), 8 (OC), 4 (YVR), 8 (OVR)) to an ex-vivo, simulated Western diet via intraluminal infusion for 40 minutes prior to assessment of EDD to ACh. This

ex-vivo challenge comprised warmed physiological saline containing 8mM glucose (in addition to 5mM glucose already present in physiological saline, Sigma Aldrich Corp.) and 300 μ M palmitate (Sigma Aldrich Corp.), two of the major metabolites present upon consumption of a Western-style diet high in saturated fat and sugar. These concentrations were selected to simulate those reported in the circulation of rodents following chronic consumption of Western-style diets (Buettner et al. 2007; Gregoire et al. 2002; Bailey-Downs et al. 2013; Symons et al. 2009). The impairments in peak EDD and AUC induced by this ex-vivo simulated Western diet were determined as the relative reduction in peak EDD or AUC in the presence versus absence of ex-vivo Western diet ($[\text{PeakEDD}_{\text{ACh}} - \text{PeakEDD}_{\text{WD}} / \text{PeakEDD}_{\text{ACh}}] \times 100$; $[\text{AUC}_{\text{ACh}} - \text{AUC}_{\text{WD}} / \text{AUC}_{\text{ACh}}] \times 100$).

MtROS mediation of ex-vivo simulated Western diet stress

To determine the role of mtROS in mediating the effects of the ex-vivo simulated Western diet, arteries were treated with the ex-vivo simulated Western diet (as above) in the simultaneous acute, ex-vivo presence of the mitochondria-specific antioxidant MitoQ (1.0 μ M, Antipodean Pharmaceuticals, Inc., Menlo Park, CA, USA; gifted by Michael P. Murphy) to scavenge mtROS for 40 minutes prior to assessment of EDD to ACh (Gioscia-Ryan et al. 2014).

Arterial mitochondrial superoxide production

Mitochondrial superoxide production was assessed using electron paramagnetic resonance spectroscopy, as previously described (Fleenor et al. 2012; Gioscia-Ryan et al. 2014; LaRocca et al. 2012). Briefly, 2mm segments of thoracic aorta were dissected free of surrounding tissue and then incubated for one hour at 37° C in Krebs-HEPES buffer with the spin probe MitoTEMPO-H (0.5 mM, Enzo Life Sciences, Inc., Farmington, NY, USA), which specifically detects superoxide produced by mitochondria (Dikalov et al. 2011). Following the

incubation period, the amplitude of the signal was measured on an MS300 X-band EPR spectrometer (Magnettech GmbH, Berlin, Germany) with settings as follows: center field, 3350G; sweep, 80G; microwave modulation, 3000 mG; microwave attenuation, 7dB. Values are expressed relative to the mean of the young control group.

Arterial protein expression

Because the carotid arteries, large elastic arteries, were used for ex-vivo measurement of endothelial function as described above, arterial protein expression was assessed using standard Western blotting procedures in homogenate from thoracic aorta, a representative large elastic artery, as performed previously (Durrant et al. 2009; Rippe et al. 2010). To confirm that aortic expression of our proteins of interest was similar to that of the carotid arteries, we assessed expression of a subset of proteins in both carotid arteries and aorta from a separate group of young and old animals (n=6/group). Following homogenization in RIPA buffer, 15 µg of protein were loaded into 4-12% polyacrylamide gels for separation by electrophoresis (Criterion System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were transferred onto nitrocellulose membranes (Trans-Blot Turbo System, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and incubated overnight with the following primary antibodies: peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α , 1:1000, Novus Biologicals, USA), NAD-dependent deacetylase sirtuin-3 (SIRT-3, 1:500, AbCam, Inc.), catalase (1:1000, AbCam, Inc.) heat shock protein 90 (Hsp90, 1:1000, Enzo Life Sciences, Inc.), Total OXPHOS Rodent Antibody Cocktail (containing antibodies against Complex I subunit NDUF8, Complex II –30k, Complex III Core protein 2, Complex IV subunit I, and Complex V alpha subunit; 1:250, Novus Biologicals, USA), Fis1 (TTC11; 1:500, Novus Biologicals, USA), Mitofusin 2 (Mfn2; 1:500, AbCam, Inc.), and beta actin (normalizer, 1:2000, Cell Signaling Technology, Inc.).

Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) and enhanced chemiluminescence (ECL) substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) on a digital acquisition system (ChemiDoc-It; UVP, Inc., Upland, CA, USA). Individual protein expression values were quantified using Image J software (Bethesda, MD, USA) (Schneider et al. 2012) and normalized to beta actin to control for differences in protein loading. Values for a single blot were expressed relative to the mean of the young control group. Western blots were run in duplicate and results for each animal were averaged. Representative images of individual proteins were obtained from the same blots using identical imaging conditions.

Statistics

All statistical analyses were performed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, USA). Data were assessed for the presence of outliers (Grubb's test), normality and homogeneity of variance prior to statistical analyses. Group differences were determined for the following variables using one-way ANOVA: Peak EDD and EDD AUC for each dose-response condition (ACh alone, rotenone, simulated WD, simulated WD+MitoQ); relative impairment induced by rotenone; relative impairment induced by simulated WD; arterial mitochondrial superoxide production; and arterial protein expression. Within-group differences in peak EDD and EDD AUC under different treatment conditions (e.g., in the presence vs. absence of MitoQ) were determined using a repeated-measures analysis of variance, with group as the between factor and treatment (simulated WD, simulated WD+MitoQ, etc.) as the repeated factor. When overall group or treatment differences were detected, specific pair-wise differences were identified with Fisher's least significant difference post-hoc tests (normally-distributed variables) or Games-Howell post-hoc tests (non-normally-distributed variables). P-values <0.05

were considered statistically significant. All data are presented as mean values (SD) in the text and mean values (SEM) in figures for clarity, unless otherwise indicated.

RESULTS

Morphological characteristics and voluntary wheel running

Select morphological characteristics and running wheel activity are presented in **Table 1**. Body mass and liver mass did not differ among groups following the 10-week voluntary aerobic exercise intervention. Age-related changes in heart mass (increase), visceral fat mass (decrease) and muscle mass (decrease) were unaltered by the late-life voluntary aerobic exercise intervention, similar to previous reports (Durrant et al. 2009, Lesniewski et al. 2013). Carotid artery diameter was increased with aging and with voluntary aerobic exercise. Voluntary running activity was significantly greater in young versus old mice, consistent with previous reports, and the average daily running activity in the old voluntary running group was similar to levels previously reported by our laboratory to improve arterial function (Durrant et al. 2009; Lesniewski et al. 2013).

Voluntary aerobic exercise reverses vascular endothelial dysfunction and normalizes arterial mitochondrial superoxide production in old mice

In order to examine the effects of voluntary aerobic exercise on arterial resilience, we first confirmed that voluntary wheel running had similar effects on baseline endothelial function as have been reported previously (Durrant et al. 2009). Peak EDD (**Figure 1B**) and EDD AUC (**Figure 1C**) were significantly lower in arteries of old control compared to young control mice. Consistent with our previous report (Durrant et al. 2009), 10 weeks of voluntary wheel running late in life completely restored endothelial function in old animals to levels similar to those of young animals, whereas the exercise intervention had no further effect on endothelial function in

arteries from young mice. There were no differences in endothelium-independent dilation in response to SNP (data not shown), indicating that the differences among groups were specific to the endothelium and not a result of changes in responsiveness of the vascular smooth muscle to NO, consistent with previous reports (Durrant et al. 2009; Lesniewski et al. 2013).

We also observed that the age-associated impairment in endothelial function was accompanied by a significant increase in basal arterial mitochondrial superoxide production, which was normalized by voluntary wheel running (**Figure 1D**). These results suggest that normalization of the age-related increase in arterial mitochondrial oxidative stress may contribute to improvements in arterial endothelial function induced by voluntary aerobic exercise.

Voluntary aerobic exercise increases arterial resilience to acute mitochondria-specific stress

Acute treatment with rotenone to induce mitochondrial oxidative stress slightly impaired peak EDD (**Figure 2A**) and EDD AUC (**Figure 2B**) in arteries of young control mice, whereas this acute mitochondrial stress caused substantial, further impairments in EDD in old control mice; importantly, the relative impairment in EDD in old control mice was significantly greater than in young control mice (**Figure 2C**). Voluntary wheel running improved arterial resilience to this stressor, such that rotenone had no significant effect in arteries of young or old voluntary wheel-running mice.

These results suggest that aging is accompanied by increased vulnerability of arteries to an acute mitochondria-specific stressor, and that voluntary aerobic exercise increases the ability of arteries to resist this stress.

Voluntary aerobic exercise increases arterial resilience to simulated metabolic stress

Treatment with ex-vivo simulated Western diet reduced peak EDD (**Figure 3A**) and EDD AUC (**Figure 3B**) in arteries of both young and old control mice, but the degree of impairment

was significantly greater in old control mice (**Figure 3C**). Simultaneous acute, ex-vivo treatment with the mitochondria-specific antioxidant MitoQ attenuated the simulated Western diet-induced reductions in EDD in young and old control mice, indicating that excessive mtROS contributed to the impairment induced by this stressor.

Voluntary wheel running improved arterial resilience to ex-vivo simulated Western diet in old mice, such that the reductions in peak EDD and EDD AUC induced by the simulated Western diet stressor in old exercising mice were less pronounced and not different in magnitude compared to those of young control and young exercising mice. In contrast to what we observed in arteries from young and old control mice, simultaneous ex-vivo MitoQ treatment had no effect compared to the simulated Western diet alone in either young or old exercising mice, suggesting an absence of excessive mtROS under conditions of simulated Western diet stress in these groups.

Together, these data suggest that, in sedentary mice, exposure of arteries to ex-vivo simulated Western diet induces or exacerbates endothelial dysfunction mediated by excess mtROS, and that voluntary aerobic exercise increases arterial resilience to the mtROS-associated component of this stressor.

Voluntary aerobic exercise normalizes age-related alterations in markers of arterial mitochondrial health but not respiratory protein content

Aortic protein expression of PGC-1 α and SIRT3, key markers of mitochondrial signaling and health, was significantly lower in arteries from old control compared to young control mice (**Figure 4**). Expression of Fis1, an important mediator of mitochondrial fission that is increased in settings of mitochondrial dysregulation (Makino et al. 2010; Miller et al. 2013; Seo et al. 2010; Shenouda et al. 2011) was greater in arteries of old control versus young control mice,

whereas expression of the key fusion mediator Mfn2 tended to be lower in arteries from old versus young control mice ($p=0.1$), indicating an age-associated shift in mitochondrial dynamics toward increased fission. Age-related differences in protein expression of a subset of these markers were comparable whether assessed in the carotid arteries or aorta (**Table 2**), suggesting that arterial mitochondrial health changes similarly in these two large elastic arteries. Importantly, voluntary aerobic exercise in old mice normalized expression of PCG-1 α , SIRT3 and Fis1 (with no effect on Mfn2), and had no further effect on expression of these proteins in young mice. These results suggest that voluntary aerobic exercise reverses age-related declines in markers of arterial mitochondrial health, perhaps contributing to some of its beneficial effects on endothelial function and stress resilience.

There were no statistically-significant differences in expression of respiratory chain protein subunits from complexes I, II, III and V among groups (we were unable to resolve bands for the subunit of complex IV), indicating that arterial mitochondrial respiratory protein content was not altered with primary aging or voluntary aerobic exercise (**Table 3**).

Voluntary aerobic exercise augments markers of arterial antioxidant defense and stress response

Aortic protein expression values of the antioxidant protein catalase and stress-response protein Hsp90 were not different in old versus young control mice (**Figure 5**). However, voluntary aerobic exercise significantly increased expression of catalase and Hsp90 in arteries from both young and old mice. These results indicate that voluntary aerobic exercise augments these key mediators of cellular antioxidant defense and stress response (Arslan et al. 2006; Manoli et al. 2007; Balaban et al. 2005) in arteries, which may contribute to the improved resilience of arteries in exercise-trained mice.

DISCUSSION

The primary, novel findings of this study are that voluntary aerobic exercise improves resilience of aging arteries to acute, mtROS-mediated stress, and that this is accompanied by normalization of basal arterial mtROS production and improvements in arterial mitochondrial health and cellular antioxidant defense and stress response proteins.

Previous studies in our laboratory (Durrant et al. 2009; Lesniewski et al. 2013) reported that late-life voluntary aerobic exercise restores baseline vascular endothelial function in old mice and we replicated this in the present study to confirm the effectiveness of the exercise intervention. These studies identified normalization of arterial oxidative stress as a key mechanism underlying the beneficial effects of voluntary aerobic exercise. Here, we extend these findings by specifically examining the role of mitochondria-derived oxidative stress and its potential amelioration by voluntary aerobic exercise. In line with a recent study in our laboratory (Gioscia-Ryan et al. 2014), we show that age-related vascular endothelial dysfunction is accompanied by elevated arterial mitochondrial superoxide production. Importantly, voluntary aerobic exercise normalized mitochondrial superoxide production in arteries of old mice, suggesting exercise-induced reductions in arterial mitochondrial oxidative stress may contribute to improvements in vascular endothelial function.

Our findings further extend previous work by demonstrating that, in addition to improving baseline vascular endothelial function, voluntary aerobic exercise restores arterial resilience in old mice. Consistent with our previous report (Gioscia-Ryan et al. 2014), we observed that acute treatment with rotenone, a mitochondria-specific inhibitor that induces mitochondrial superoxide production (Li et al. 2003; Weir et al. 1991), impairs carotid artery endothelial function in old mice to a greater degree than arteries from young mice. These results

provide evidence that mitochondrial health is altered in aging arteries such that arteries are more vulnerable to a mitochondria-specific challenge. Importantly, in the present study, we show that voluntary aerobic exercise completely restores the ability of aged arteries to withstand this acute mitochondrial stress.

In addition to a mitochondria-specific challenge, we also examined arterial resilience in response to a simulated Western diet stressor composed of ex-vivo glucose and palmitate, major nutrients present in the circulation with consumption of a high-fat, high-sugar Western diet. Ex-vivo simulated Western diet exposure caused a more substantial, further impairment in endothelial function in old sedentary mice versus young sedentary and young and old exercising mice. When considered from a translational perspective, it is important to note that although the ex-vivo simulated Western diet slightly impaired endothelial function in young sedentary and young and old exercising mice, the resulting level of function in these groups (peak EDD ~65-80%) was still substantially greater than that of old sedentary mice, in which the combined effects of aging and the ex-vivo simulated Western diet stressor resulted in dramatically reduced endothelial function (peak EDD ~45%).

The increase in arterial susceptibility to ex-vivo simulated Western diet with aging is similar to what is observed in a setting of chronic, in vivo Western diet consumption in mice. Old mice consuming Western diet for 10 weeks display significantly reduced endothelial function even compared to old mice consuming normal chow, and the magnitude of impairment induced by chronic Western diet consumption in old mice is much greater than the modest impairment observed in young mice consuming Western diet (Lesniewski et al. 2013). Simultaneous aerobic exercise, in the form of voluntary wheel running, completely preserves endothelial function in young and old mice chronically consuming Western diet (Lesniewski et

al. 2013). However, in the present study, we observed that although voluntary wheel running significantly improved arterial resilience to acute challenge with ex-vivo simulated Western diet, it did not confer complete protection against this stressor. This difference in exercise-induced arterial protection between studies may be due to the acute, ex-vivo nature of the simulated Western diet stress in the present study versus chronic Western diet consumption.

The observation that ex-vivo simulated Western diet exposure acutely impairs endothelial function is also consistent with clinical data that show reduced flow-mediated dilation following consumption of single high-fat meals (Bae et al. 2001; Fard et al. 2000; Nicholls et al. 2006; Plotnick et al. 2003) in heterogeneous populations that include older adults. Although data comparing arterial resilience in young versus older subjects is currently lacking, cross-sectional data support the premise that arterial resilience declines with age. Endothelial function is lower in older adults with moderately elevated fasting blood glucose or LDL cholesterol versus their age-matched sedentary peers with normal blood glucose or LDL levels (DeVan et al. 2013; Walker et al. 2009), whereas—in support of our finding that exercise improves arterial resilience in mice—endothelial function is preserved at levels similar to young adults in older habitually exercising adults with both normal and elevated blood glucose or LDL cholesterol (DeVan et al. 2013; Walker et al. 2009).

Simultaneously treating arteries from young and old sedentary mice with ex-vivo MitoQ abolished the impairment induced by simulated Western diet exposure, suggesting that the effects of this stressor were acutely mediated by excess mtROS. That the effects of this stressor may involve disruption of mitochondrial health is not unexpected, as glucose and palmitate independently increase mtROS production in vitro (Paneni et al. 2012; Inoguchi et al. 2000; Du et al. 2000; Yamagishi et al. 2001, Koziel et al. 2015; Kumar et al. 2015; Gao et al. 2011; Lu et

al. 2013). Additionally, arterial mitochondrial dysfunction is an important feature of metabolic diseases such as diabetes, in which arteries are chronically exposed to high circulating levels of glucose and lipids (Giacco & Brownlee 2010; Makino et al. 2010; Shenouda et al. 2011).

Voluntary aerobic exercise conferred protection against ex-vivo simulated Western diet exposure in old mice, such that the impairment in endothelium-dependent dilation in arteries from old voluntary wheel running mice was much smaller in magnitude than that of old control mice, and not different from young control or young voluntary wheel running mice. This exercise-induced increase in arterial resilience appears to be primarily mediated by improved mitochondrial health, as simultaneous ex-vivo treatment with MitoQ had no effect on the response to simulated Western diet exposure in young or old exercising mice. However, this stressor did result in a slight reduction in EDD in both exercising groups that was unaffected by MitoQ, suggesting the involvement of additional mechanisms in the impairment induced by simulated Western diet.

In line with normalization of mtROS production and increased arterial resilience to mtROS-mediated challenges, our results also demonstrate that voluntary aerobic exercise normalizes key indicators of arterial mitochondrial health in old mice. Despite relatively low abundance (Blouin et al. 1977) and minimal respiratory activity (Dai et al. 2012; Kluge et al. 2013; Dromparis & Michelakis 2013), arterial mitochondria play a vital role in maintaining arterial function, presumably via other roles involving intra- and extra-cellular signaling (Dai et al. 2012; Kluge et al. 2013; Dromparis and Michelakis 2013, Widlansky & Guterman 2011). However, arterial mitochondrial health and quality control decline with aging and in disease models of hypertension, NO deficiency, atherosclerosis, diabetes and metabolic syndrome (Gioscia-Ryan et al. 2014; LaRocca et al. 2014; Keller et al. 2015; Knaub et al. 2013; Makino et

al. 2010; Miller et al. 2013; Shenouda et al. 2011; Young et al. 2004). In the present study we observed age-related declines in PGC-1 α and SIRT3, key regulators of mitochondrial biogenesis, health and antioxidant defenses (Bell & Guarente 2011; Bause & Haigis 2013; Handschin 2009), as well as a shift in mitochondrial dynamics favoring increased mitochondrial fission (e.g., increased Fis1 and decreased Mfn2), a characteristic of mitochondrial dysfunction (Makino et al. 2010; Miller et al. 2013; Seo et al. 2010; Shenouda et al. 2011). Similar changes in these markers have been observed in models of disease and arterial dysfunction and with aging (Gu et al. 2014; Knaub et al. 2013; Miller et al. 2013). It is well known that exercise improves mitochondrial health and signaling in tissues such as skeletal muscle, promoting increased mitochondrial quality (Koltai et al. 2012; Hood et al. 2011; Meshnikova et al. 2006; Irrcher et al. 2003; Ljubicic et al. 2010; Handschin 2009). Previous studies investigating the effects of exercise on arterial mitochondria indicate that although exercise induces favorable changes in arterial mitochondrial health in young, healthy animals, the arterial mitochondrial response to exercise is impaired with vascular and metabolic disease (Keller et al. 2015; Knaub et al. 2013; Miller et al. 2013; Young et al. 2004). One recent study reported increases in PGC-1 α and mitochondrial respiratory protein content in aged rats following an exercise intervention (Gu et al. 2014). Our observation that voluntary aerobic exercise normalized age-related changes in PGC-1 α , SIRT-3 and Fis1 provide further evidence that arterial mitochondria of aged animals can adapt to exercise training and suggest that exercise-induced improvements in arterial mitochondrial health may be an important aspect of exercise-mediated vascular protection.

Finally, we also observed that voluntary aerobic exercise augments arterial expression of heat shock protein 90 and catalase in both young and old mice. Hsp are ubiquitously-expressed and highly-inducible chaperone proteins that are activated in response to a variety of cellular

stressors (Arslan et al. 2006; Fiuza-Luces et al. 2013; Noble et al. 2008) and interact with mitochondria to mediate cell survival pathways (Manoli et al. 2007; Noble et al. 2008). In the vasculature, Hsp90 interacts with endothelial nitric oxide synthase (eNOS) to promote NO production (García-Cardena et al. 1998). Exercise is a potent stimulus for induction of Hsp and increases localization of Hsp in the coronary and skeletal muscle vasculature (Milne et al. 2012; Rinaldi et al. 2006), and this is thought to contribute to the protective effects of aerobic exercise in these tissues (Rinaldi et al. 2006; Quindry 2012; Milne et al. 2012). Although few studies have specifically examined the effects of aerobic exercise on Hsp induction in the systemic vasculature, resistance training-induced increases in femoral artery Hsp90 were associated with improvements in eNOS activity and arterial function in young and old rats (Harris et al. 2010). In the present study, we observed that arterial expression of Hsp90 was significantly increased in both young and old mice following the voluntary aerobic exercise intervention.

Catalase is an antioxidant enzyme responsible for facilitating the decomposition of hydrogen peroxide, the dismutation product of superoxide, into water. Although superoxide is the primary species of ROS produced by mitochondria, the more stable hydrogen peroxide is thought to be the main transducer of many physiological and pathophysiological effects of mtROS (Collins et al. 2012; Murphy 2009). Acute and chronic aerobic exercise training induce increases in catalase in the heart and vasculature and these adaptations are thought to contribute to reduced progression of vascular disease and increased cardiac resilience to acute ischemia/reperfusion injury (Gielen et al. 2010; Kavazis et al. 2008; Meilhac et al 2001; Sindler et al. 2013). Additionally, previous studies from our laboratory and others have demonstrated increases in content and activity of other antioxidant enzymes, including the key mitochondrial antioxidant manganese superoxide dismutase, in arteries following aerobic exercise interventions

(Durrant et al. 2009; Fukai et al. 2000; Rush et al. 2000). The exercise-induced increase in arterial catalase that we observed in this study is consistent both with previous reports and with our finding of increased resilience to mtROS-mediated acute stress. Our results suggest that aerobic exercise-induced augmentation of cellular stress response pathways and antioxidant defenses may contribute to vascular protective effects of voluntary aerobic exercise.

Considerations and future directions

The primary aim of this study was to examine the effects of primary aging on arterial resilience to acute stress and to determine whether voluntary aerobic exercise is an intervention that increases arterial resilience. The stressors we employed in this study were acutely applied to arteries ex-vivo for a short (~40 minutes) period of time. We designed our ex-vivo simulated Western diet to reflect two of the most prevalent nutrients (glucose and palmitate) that would be expected to be in contact with the vasculature in settings of chronic Western diet consumption and the concentrations used in the present study were selected to reflect levels commonly measured in the circulation of rodents ingesting Western-style diets (Buettner et al. 2007; Gregoire et al. 2002; Bailey-Downs et al. 2013; Symons et al. 2009). This stressor induced impairments in endothelial function, particularly in old sedentary mice, and we observed a similar age-related increase in susceptibility to simulated Western diet stress compared to chronic Western diet consumption (Lesniewski et al. 2013). However, we recognize that this simple model of ex-vivo challenge does not recapitulate all the elements of a Western diet and that acute stressors may not reflect all aspects of chronic challenges such as persistently elevated blood lipids or glucose or chronic consumption of a Western diet. For example, although excessive levels of mtROS contributed acutely to the stressor-induced dysfunction we observed, it is unlikely that the impairments were due to changes in mitochondrial DNA damage or gene

expression, alterations that might be expected as a consequence of chronic mtROS elevations. Therefore, future studies are needed to examine arterial resilience to stress following in vivo acute and chronic Western diet consumption.

Although our results with acute MitoQ treatment indicate that suppression of endothelial function by the simulated WD stressor is primarily mediated by mtROS, there remained a small amount of impairment in arteries of the exercise-trained animals following acute MitoQ treatment, suggesting involvement of a non-mtROS mechanism. Previous work employing ex-vivo glucose and palmitate demonstrate that these compounds induce oxidative stress, a major source of which is increased production of mitochondria-derived superoxide (Gao et al. 2011; Kumar et al. 2015; Lu et al. 2013; Koziel et al. 2015; Yuzefovych et al. 2010). However, these compounds have also been reported to stimulate superoxide production via non-mitochondrial pathways, including activation of the pro-oxidant enzyme NADPH oxidase (Chinen et al. 2007) or the renin-angiotensin system (Wantanabe et al. 2005). Experimental paradigms involving longer (e.g., 8-24 hour) exposures to hyperglycemia or palmitate suggest that these stressors can also induce changes in gene expression or post-transcriptional modifications, including suppression of AMPK signaling and induction of NF κ -B mediated pro-inflammatory signaling, that may act to suppress endothelial function (Cacicedo et al. 2004; Fard et al. 2000; Staiger et al. 2004; Wu et al. 2007). Importantly, our results indicate that voluntary aerobic exercise improves arterial resilience to the mitochondria-specific oxidative stress induced by simulated WD exposure.

In addition to the exercise-induced augmentation of antioxidant defenses we observed that may allow arteries to maintain vasodilatory function in the face of acute mtROS challenges, it is also important to consider the possibility that exercise may induce adaptations that enable

arteries to dilate in response to mtROS, specifically hydrogen peroxide, which is emerging as a critical vasodilator in the coronary and resistance vasculature (Beyer et al. 2014; Durand et al. 2015; Liu et al. 2003, Miura et al., 2003, Muller-Delp et al. 2012). Age and exercise have been reported to influence the relative contribution of hydrogen peroxide to vasodilatory responses in resistance arterioles (Beyer et al. 2014; Muller-Delp et al. 2012; Sindler et al. 2013), and the exercise-induced enhancement of aortic catalase expression that we observed suggests an adaptation to elevated hydrogen peroxide levels. Thus, future studies are warranted to determine if hydrogen peroxide-mediated dilation may have contributed to the maintenance of vasodilation in the presence of acute mtROS stressors that we observed in the arteries from exercise-trained mice.

We observed improvements in protein markers of arterial mitochondrial health with voluntary aerobic exercise that were consistent with previous studies in young, healthy animals. However, in contrast to some previous reports investigating the effects of exercise on arterial mitochondria (Gu et al. 2014; Keller et al. 2015; Knaub et al. 2013; Miller et al. 2013; Young et al. 2004; Park et al. 2016) we did not observe an increase in arterial mitochondrial respiratory protein content with voluntary aerobic exercise. The lack of increase in mitochondrial protein content that we observed may be attributable to the nature of the exercise intervention, as voluntary wheel running—an intermittent stimulus—is distinctly different than the forced, continuous treadmill and swimming exercise paradigms employed in previous investigations. However, our results suggest that improvements in upstream regulators of arterial mitochondrial health induced by exercise can occur independently of changes in mitochondrial protein content and underscore the importance of mitochondrial quality control in arterial tissue. Indeed, the functional roles of vascular mitochondria may be more contingent on mitochondrial quality

versus mitochondrial content. Mitochondria perform vital signaling functions in the vasculature (Dai et al. 2010; Dromparis & Michelakis 2013; Kluge et al. 2013) that depend on maintenance of an interconnected network of mitochondria via a balance of mitochondrial fission and fusion, as well as mitophagy (Mammucari & Rizzuto 2010; Seo et al. 2010; Weber & Reichert 2010). Thus, preservation of mitochondrial quality is critical in the vasculature, whereas an increase in mitochondrial respiratory protein content might be expected to confer little benefit to vascular cells that rely sparingly on aerobic metabolism. Although the exercise-induced improvements in markers of mitochondrial health we observed strongly suggest that voluntary aerobic exercise improves arterial mitochondrial function with aging, future work is needed to confirm whether the observed effects of aerobic exercise extend to more comprehensive indices of intact mitochondrial function in arteries.

We assessed markers of mitochondrial health in whole large elastic arteries and thus are not able to distinguish changes that may be specific to endothelial cells versus vascular smooth muscle cells (VSMCs). However, given that vascular mitochondria are particularly critical for intra- and inter- cellular signaling functions (Dai et al. 2010; Dromparis & Michelakis 2013; Kluge et al. 2013), mitochondria in both vascular cell types are likely important for regulation of arterial endothelial function. In addition to our finding that preserved arterial resilience with exercise is accompanied by improvements in whole artery mitochondrial health, previous work has also demonstrated that alterations in whole artery and VSMC (Gioscia-Ryan et al. 2014; Gu et al. 2014; Keller et al. 2015; Knaub et al. 2013; LaRocca et al. 2013; Miller et al. 2013; Park et al. 2016) mitochondrial health with aging, disease and exercise are accompanied by corresponding changes in endothelial function.

In conclusion, age-associated vascular endothelial dysfunction is accompanied by reductions in arterial resilience and mitochondrial health (elevated mtROS production, reduced markers of mitochondrial biogenesis/signaling, altered fission/fusion dynamics). Ten weeks of voluntary aerobic exercise improved arterial resilience to acute mitochondria-specific and ex-vivo simulated Western diet stressors, normalized age-related alterations in arterial mitochondrial health, and augmented arterial markers of antioxidant defense and cellular stress response. Overall, our results highlight the importance of healthy mitochondria for maintenance of arterial function and resilience with aging, and identify voluntary aerobic exercise as a later-life intervention that improves arterial mitochondrial health as well as resilience.

ACKNOWLEDGEMENTS

The authors would like to thank James Richey for his research support.

FUNDING

This work was supported by NIH grants AG047784 (RGR); AG000279 (RGR); HL107120-04 (DRS); AG013038 (DRS)

Table 1. Select morphological characteristics and voluntary running wheel activity

	YC	OC	YVR	OVR
Body mass (g)	32.15 (2.94)	30.71 (3.12)	32.08 (4.0)	28.95 (1.77)
Heart mass (mg)	152.9 (10.3)	193.0 (18.2)*^	144.6 (11.6)	216.5 (4.6)*^
Liver mass (g)	1.81 (0.15)	1.65 (0.24)	1.58 (0.33)	1.69 (0.16)
Quadriceps mass (mg)	198.4 (36.3)	143.3 (21.5)*^	190.3 (16.8)	138.4 (9.23)*^
Visceral fat mass (mg)	82.49 (48.1)	24.4 (13.3)*^	85.6 (60.2)	12.2 (6.8)*^
Carotid artery diameter (μ m)	418 (18) [#]	448 (20) ^{*#}	451 (20) [*]	470 (40) [*]
Running activity (km/day)	n/a	n/a	19.37 (14.0)	3.13 (1.80) [^]

Data are presented as means (SD), n=10-12/group. Abbreviations: YC, young control mice; OC, old control mice; YVR, young voluntary wheel running mice; OVR, old voluntary wheel running mice. * p<0.05 vs. YC; ^ p<0.05 vs. YVR, # p<0.05 vs. OVR

Table 2. Protein expression of mitochondrial health markers in carotid arteries versus aorta of young and old mice

	YCar	OCar	YAor	OAor
PGC-1 α (AU)	1.0 (0.14)	0.73 (0.15)	1.0 (0.12)	0.71 (0.15)
SIRT3 (AU)	1.0 (0.07)	0.70 (0.12)	1.00 (0.05)	0.80 (0.11)
Mfn2 (AU)	1.0 (0.33)	0.73 (0.35)	1.00 (0.36)	0.75 (0.18)

Data are presented normalized to beta actin and relative to the mean of the young group for each type of artery and are shown as mean (SEM), n=6/group. Abbreviations: YCar, carotid arteries from young control mice; OCar, carotid arteries from old control mice; YAor, aorta from young control mice, OAor, aorta from old control mice.

Table 3. Aortic protein expression of mitochondrial respiratory complex proteins

	YC	OC	YVR	OVR
Complex I (AU)	0.96 (0.32)	1.0 (0.65)	0.78 (0.24)	1.04 (0.67)
Complex II (AU)	0.96 (0.41)	0.85 (0.52)	0.79 (0.30)	0.98 (0.66)
Complex III (AU)	0.97 (0.99)	1.04 (0.77)	0.78 (0.35)	0.96 (0.63)
Complex V (AU)	1.07 (0.52)	0.85 (0.36)	0.95 (.47)	0.99 (0.84)

Data are presented normalized to beta actin and relative to the mean of the young control group and are shown as means (SD), n=6-8/group. Abbreviations: YC, young control mice; OC, old control mice; YVR, young voluntary wheel running mice; OVR, old voluntary wheel running mice. No significant differences were detected among groups.

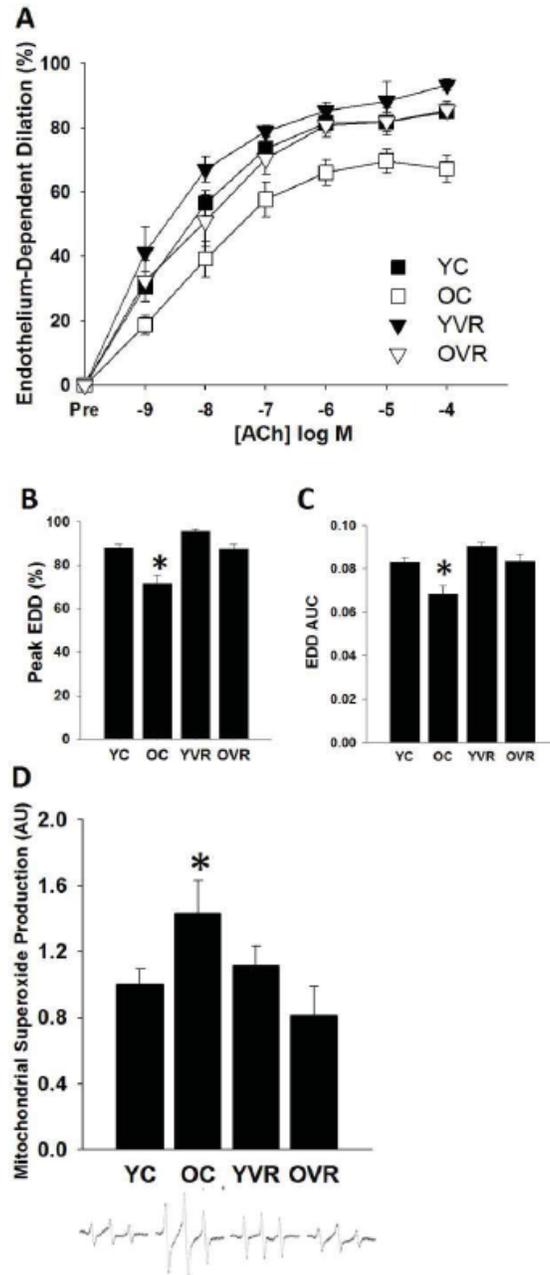


Figure 1. Voluntary aerobic exercise restores endothelium-dependent dilation and normalizes mitochondrial superoxide production in old mice

Endothelium-dependent dilation (EDD) dose-response (A) to acetylcholine (ACh), peak dilation (B), and EDD AUC (C) in carotid arteries and aortic mitochondria-specific superoxide production (D) in young control (YC), old control (OC), young voluntary wheel running (YVR) and old voluntary wheel running (OVR) mice. Representative EPR spectra presented below panel D. Data are presented as mean (SEM), n=10-12 per group. * p<0.05 vs. YC. Peak EDD and EDD AUC data are shown again in Figures 2 and 3 for clarity of interpretation of within-group changes in EDD in the presence of acute stressors.

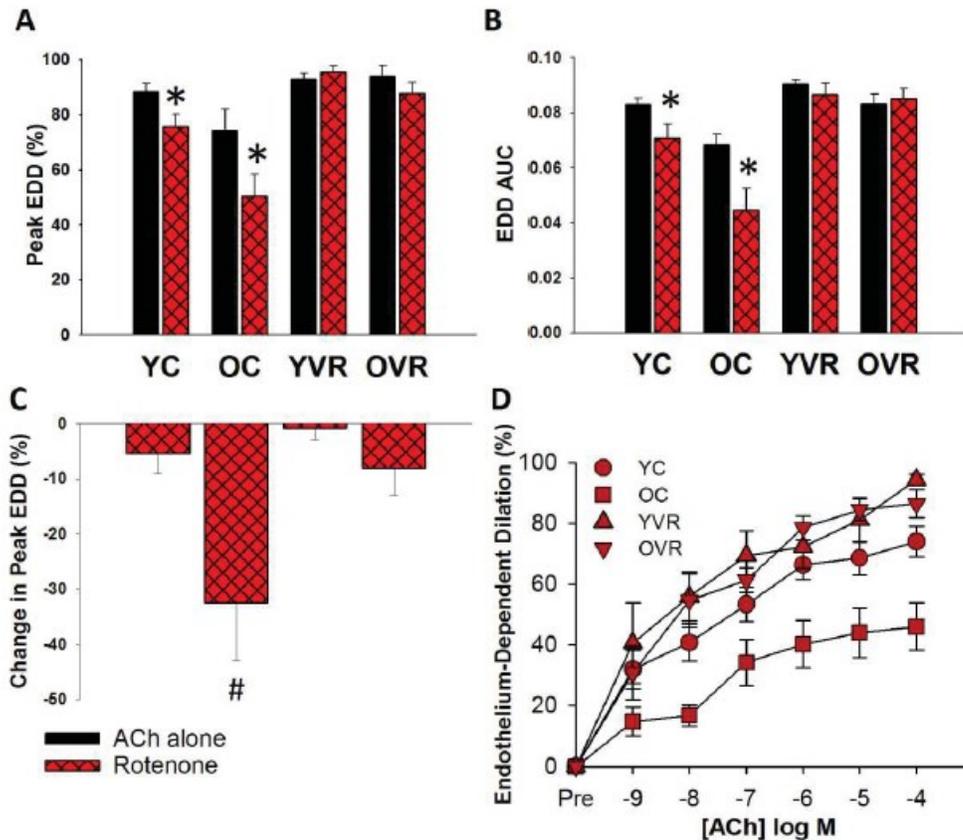


Figure 2. Voluntary aerobic exercise increases arterial resilience to acute mitochondria-specific stress

A and B: Peak endothelium-dependent dilation (EDD) and EDD AUC to acetylcholine (ACh) alone (black bars, shown again here for clarity) and in the presence of rotenone (red hashed bars) in carotid arteries from young control (YC), old control (OC), young voluntary wheel running (YVR) and old voluntary wheel running mice (OVR). C: Relative reduction in peak EDD in the presence vs. absence of rotenone in arteries from YC, OC, YVR and OVR mice. D: EDD dose-response curves to ACh in the acute presence of rotenone in carotid arteries from YC, OC, YVR and OVR mice. Data are presented as mean (SEM), n=5-8/group. * p<0.05 within-group versus ACh alone, # p<0.05 vs. all other groups.

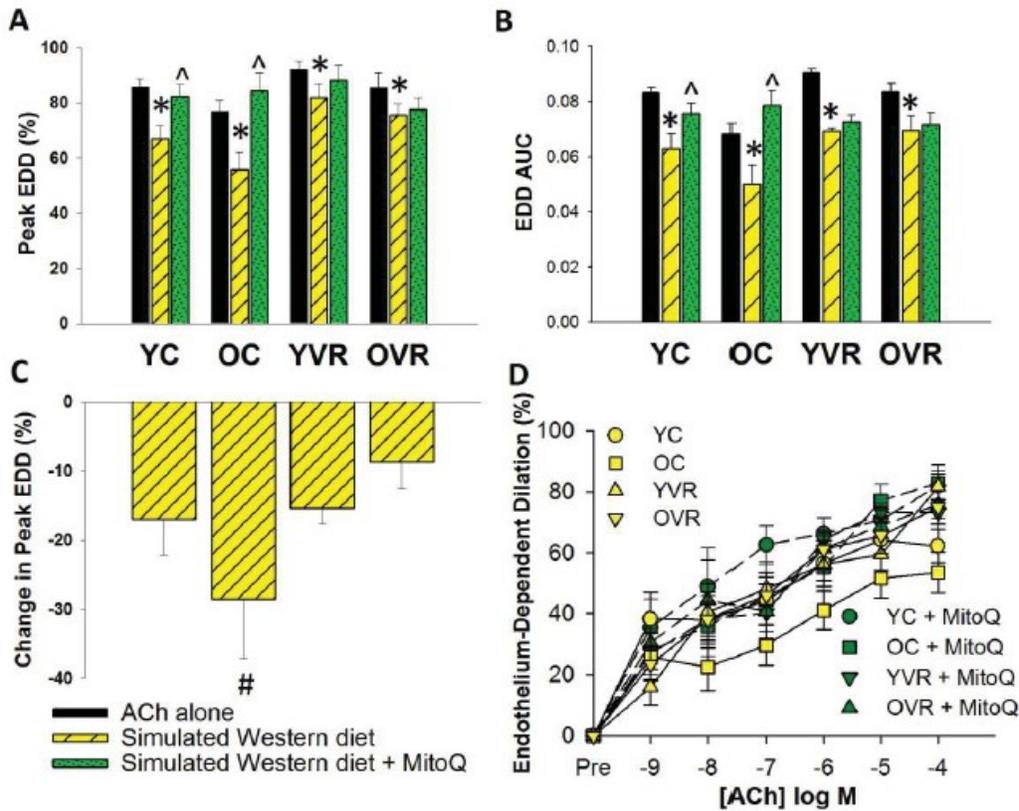


Figure 3. Voluntary aerobic exercise increases arterial resilience to acute simulated Western diet stress

A and B: Peak endothelium-dependent dilation (EDD) and EDD AUC to acetylcholine (ACh) alone (black bars, shown again here for clarity), in the presence of simulated Western diet (yellow hashed bars), and simulated Western diet + MitoQ (green hashed bars) in carotid arteries from young control (YC), old control (OC), young voluntary wheel running (YVR) and old voluntary wheel running mice (OVR). C: Relative reduction in peak EDD in the presence vs. absence of simulated Western diet in arteries from YC, OC, YVR and OVR mice. D: EDD dose-response curves to ACh in the acute presence of simulated Western diet (yellow symbols with solid lines) and simulated Western diet + MitoQ (green symbols with dashed lines) in carotid arteries from YC, OC, YVR and OVR mice. Data are presented as mean (SEM), n=4-8/group. * p<0.05 within-group versus ACh alone, [^] p<0.05 within-group versus simulated Western diet, [#] p<0.05 vs. all other groups.

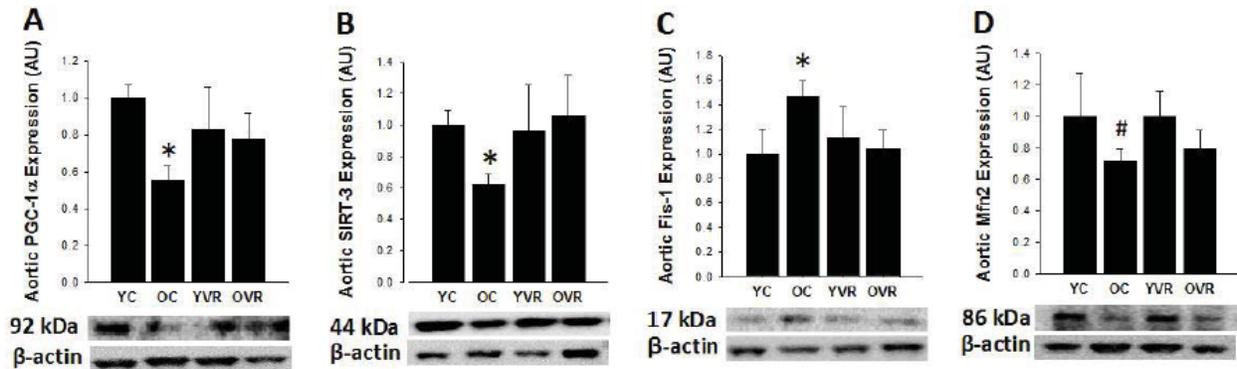


Figure 4. Voluntary aerobic exercise restores markers of arterial mitochondrial health in old mice

Protein expression of PGC-1 α (A), SIRT-3 (B), Fis1 (C), and Mfn2 (D) in arteries from young control (YC), old control (OC), young voluntary wheel running (YVR) and old voluntary wheel running (OVR) mice. Representative images are presented below each panel with corresponding images of normalizer (beta actin) taken from the same region of the same blot. Data are presented normalized to beta actin and relative to the mean of the YC group as mean (SEM), n=6-8/group. * p<0.05 vs. YC, # p=0.1 vs. YC.

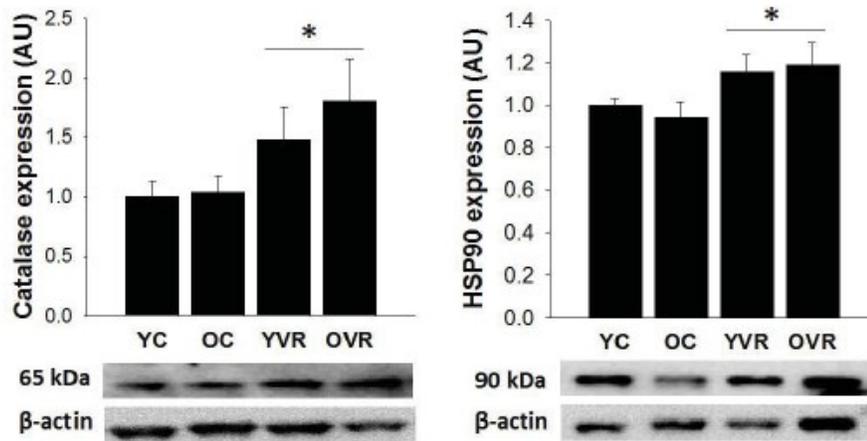


Figure 5. Voluntary aerobic exercise augments arterial markers of antioxidant defense and stress resistance

Protein expression of catalase (A) and Hsp90 (B) in arteries from young control (YC), old control (OC), young voluntary wheel running (YVR) and old voluntary wheel running (OVR) mice. Representative images are presented below each panel with corresponding images of normalizer (beta actin) taken from the same region of the same blot. Data are presented normalized to beta actin and relative to the mean of the YC group as mean (SEM), n=6-8/group. * p<0.05 vs. YC and OC.

CHAPTER V CONCLUSION

The purpose of this dissertation was to determine the role of arterial mitochondrial oxidative stress in mediating age-related arterial dysfunction and to investigate the therapeutic potential of mitochondria-targeted strategies to improve or preserve arterial function with age.

Primary arterial aging in mice was characterized by impaired nitric oxide-mediated endothelium-dependent dilation, which was accompanied by elevated levels of arterial mitochondrial oxidative stress and adverse changes to markers of mitochondrial health/homeostasis. Acute *ex-vivo* and chronic *in vivo* treatment with the mitochondria-targeted antioxidant MitoQ to reduce mitochondrial oxidative stress completely restored endothelial function and (in the case of chronic *in vivo* supplementation) normalized age-related alterations in mitochondrial health.

Arterial aging in mice was also characterized by elevated large elastic artery stiffness, associated with increased arterial collagen content and reduced arterial elastin content. Chronic *in vivo* supplementation with MitoQ reduced arterial stiffness to levels similar to those of young, healthy mice, and attenuated the age-related decline in arterial elastin content. Together, the results of these studies indicate that mitochondrial oxidative stress is a key mediator of age-related arterial dysfunction and highlight the therapeutic potential of mitochondria-targeted antioxidants to restore or preserve arterial function.

Another feature of primary arterial aging was reduced resilience, indicated by increased vulnerability of aged arteries to impairments induced by acute *ex-vivo* stressors, including a mtROS-specific challenge and a “simulated Western diet.” Late-life voluntary aerobic exercise, an established intervention for improving baseline arterial function as well as mitochondrial health, improved arterial resilience, such that the acute stressors induced minimal impairment in

arteries of old mice following exercise training. Improved arterial resilience with exercise was accompanied by normalization of age-associated adverse changes in markers of arterial mitochondrial health.

Collectively, the results of this dissertation indicate that mitochondria-derived oxidative stress is a key mechanism mediating age-related impairments in arterial function, and may therefore represent a promising therapeutic target for preserving arterial function, and reducing cardiovascular disease risk, with aging in humans.

Future Directions

The findings in this dissertation strongly suggest the possibility that targeting mitochondrial oxidative stress may be an effective strategy to preserve arterial function with aging in humans. Because MitoQ is now commercially available as a dietary supplement, clinical trials in middle-aged and older adults are warranted to determine whether the efficacy we observed in these preclinical studies translates to humans.

In the present studies, we assessed several protein markers of mitochondrial health and homeostasis and our results indicate that arterial mitochondrial health is altered adversely by primary aging and restored by late-life MitoQ supplementation and voluntary aerobic exercise. However, future studies should expand on these assessments of mitochondrial health markers and establish the effects of aging, MitoQ treatment, and exercise on intact arterial mitochondrial function, including mitochondrial respiratory function, fusion/fission dynamics, and calcium handling.

Changes in vascular mitochondrial oxidative stress and health have the potential to impact other signaling pathways involved in the regulation of arterial function; for example, mtROS can activate pro-inflammatory signaling that may directly impair endothelial function or

alter the regulation of structural protein turnover to promote arterial stiffness. Follow-up studies could examine the role of arterial mitochondrial oxidative stress in the regulation of the chronic, low-grade inflammation that occurs with primary aging.

Finally, primary aging is associated with declines in physiological function beyond those that occur in the vasculature, including impairments in physical and cognitive function that precede the development of disability and chronic diseases. Mitochondria are critical for maintenance of cellular function in numerous tissues, including the heart, skeletal muscle, and nervous system, all of which demonstrate physiological and mitochondrial declines with age. Therefore, mitochondria-targeted intervention strategies may have the potential to preserve physiological functions in addition to arterial function in the setting of primary aging, and future studies should determine the efficacy of such strategies on a broad range of physiological functional outcomes.

CHAPTER VI REFERENCES

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