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Mitochondria-targeted antioxidant therapy with MitoQ ameliorates aortic stiffening in old mice

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ABSTRACT

Aortic stiffening is a major independent risk factor for cardiovascular diseases, cognitive dysfunction and other chronic disorders of aging. Mitochondria-derived reactive oxygen species are a key source of arterial oxidative stress which may contribute to arterial stiffening by promoting adverse structural changes—including collagen overabundance and elastin degradation—and enhancing inflammation, but the potential for mitochondria-targeted therapeutic strategies to ameliorate aortic stiffening with primary aging is unknown. We assessed aortic stiffness (pulse-wave velocity (aPWV)), *ex-vivo* aortic intrinsic mechanical properties (elastic modulus (EM) of collagen and elastin regions), and aortic protein expression 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 μM; YMQ and OMQ) for 4 weeks. Both baseline and post-intervention aPWV values were higher in OC versus YC (post: 482 ± 21 vs. 420 ± 5 cm/sec, $p<0.05$). MitoQ had no effect in young mice but decreased aPWV in old mice (OMQ, 426 ± 20, $p<0.05$ vs. OC). MitoQ did not affect age-associated increases in aortic collagen-region EM, collagen expression, or pro-inflammatory cytokine expression, but partially attenuated age-associated decreases in elastin-region EM and elastin expression. Our results demonstrating that MitoQ reverses *in vivo* aortic stiffness in old mice suggest that mitochondria-targeted antioxidants may represent a novel, promising therapeutic strategy for decreasing aortic stiffness with primary aging and, possibly, age-related clinical disorders in humans. The de-stiffening effects of MitoQ treatment may be at least partially mediated by attenuation/reversal of age-related aortic elastin degradation.

NEW & NOTEWORTHY

We show that 4 weeks of treatment with the mitochondria-specific antioxidant MitoQ in mice completely reverses the age-associated elevation in aortic stiffness, assessed as aortic pulse-wave velocity. The de-stiffening effects of MitoQ treatment may be at least partially mediated by attenuation of age-related aortic elastin degradation. Our results suggest that mitochondria-targeted therapeutic strategies may hold promise for decreasing arterial stiffening with aging in humans, possibly decreasing the risk of many chronic age-related clinical disorders.

ABBREVIATIONS

aPWV, aortic pulse-wave velocity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-10, interleukin-10; IFN- γ, interferon-gamma; MMP, matrix metalloproteinase; mtROS, mitochondria-derived reactive oxygen species; NOX, NADPH oxidase; SOD2, manganese superoxide dismutase

INTRODUCTION

Advancing age is a primary risk factor for the development of numerous chronic degenerative diseases, which are the leading causes of morbidity and mortality in the United States and other developed nations (20, 30, 41). A key event underlying the etiology of many chronic age-related disorders is stiffening of the large elastic arteries, specifically the aorta. Elevated aortic stiffness increases the pulsatile shear and pressure experienced by the heart, blood vessels and other organs, which can have numerous pathophysiological effects contributing to the development of disease (23, 32, 34, 35, 38, 62). Indeed, aortic pulse-wave velocity (aPWV), the gold-standard measure of arterial stiffness, is a strong independent risk factor for incident cardiovascular events among older adults (34, 50) and also predicts the development of chronic kidney disease, stroke, cognitive impairment, and Alzheimer's Disease (2, 7, 18, 21, 43, 53). Current demographic trends forecast a major increase in the number of older adults in the coming decades which will be accompanied by attendant increases in disease prevalence and health care costs (19, 22, 56). As such, a top biomedical research priority is to identify strategies that prevent or reverse aortic stiffening with advancing age, as this may help prevent, reduce, or delay the development of multiple common disorders of aging.

A key mechanism underlying the development of age-related arterial stiffening may be vascular mitochondrial oxidative stress and associated excessive production of mitochondria-derived reactive oxygen species (mtROS). Mitochondria are now recognized as a primary source of arterial oxidative stress with aging and cardiovascular diseases (1, 4, 5, 16, 31, 38, 55, 61), and evidence from genetic models indicates that experimental modulation of mtROS affects large elastic artery stiffening. For example, age-related arterial stiffening, pathological remodeling, and vascular disease are accelerated in mice deficient in the mitochondrial

antioxidant protein manganese superoxide dismutase (SOD2) (61). In support of a role specifically for mitochondria-derived oxidative stress, selective deletion of a cytosolic isoform of pro-oxidant enzyme NADPH oxidase (NOX1/2)—with the mitochondrial isoform (NOX4) intact—does not prevent age-related arterial stiffening in the setting of atherosclerosis (55), implicating mtROS as a key driver of age-related arterial pathology.

Excessive levels of arterial mtROS may promote arterial stiffness via redox-related alterations in structural protein turnover, and through induction of pro-inflammatory signaling. Changes in arterial wall structure are a major mechanism by which the large elastic arteries stiffen with age (9, 17, 24, 32, 62); specific structural alterations include increased deposition of the load-bearing protein collagen and degradation and fragmentation of elastin (17, 24, 42). Oxidative stress, including that derived specifically from mitochondria, alters the activity of the enzymes involved in structural protein turnover and shifts the balance of synthesis and breakdown toward collagen deposition and elastin degradation (9, 17, 24, 38, 55, 61, 62), contributing to dysregulation of structural protein homeostasis and consequent arterial stiffening.

Mitochondria-derived ROS are also emerging as important for promoting and sustaining arterial inflammation, a hallmark of arterial aging and critical mediator of arterial stiffening (24, 38, 39, 57). A pro-inflammatory environment in the vasculature, secondary to excessive mtROS production, may contribute to arterial stiffening through many mechanisms, including induction of gene expression patterns that alter structural protein turnover, impairment of vascular endothelial function, increases in vascular smooth muscle cell tone, and further invasion of the vascular wall by pro-inflammatory mediators that also reinforce oxidative stress (24, 31, 32, 39, 57, 61).

Our laboratory recently demonstrated that treating old mice with the mitochondria-targeted antioxidant MitoQ to lower mitochondrial oxidative stress completely reversed the age-related impairment in arterial endothelial function in old mice (15). 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 decrease aortic stiffness (as assessed *in vivo* by aPWV) in old mice. To gain insight into the potential underlying mechanisms, we also assessed the collagen- and elastin-mediated contributions to intrinsic aortic stiffness (assessed *ex vivo* in aortic rings), aortic protein expression of these key structural proteins, and aortic expression of inflammatory cytokines.

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 (15, 48), were purchased from the aging colony at the National Institute on Aging at ~4 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 (15, 40, 46), mice were randomly assigned to treatment with MitoQ (250 μM; in the form of Mitoquinone mesylate adsorbed to β-cyclodextrin (~22% MitoQ by weight) from Antipodean Pharmaceuticals) (young MitoQ-treated [YMQ, ~6 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 (15). MitoQ was prepared fresh (the preparation is water-soluble) 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 following 4 weeks of MitoQ treatment by aortic pulse-wave velocity (aPWV) using Doppler ultrasound, as previously described by our laboratory (11, 28). 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 ($\Delta time_{abdominal}$ and $\Delta time_{transverse}$). aPWV was then calculated as aPWV = (physical distance between the two probes) / ($\Delta time_{abdominal}$-$\Delta time_{transverse}$) and reported in cm/sec.

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 (11, 28). 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 from the thoracic region (dissected free of surrounding connective tissue) were used to assess intrinsic aortic stiffness via wire myography, as described previously by our laboratory (6, 10, 14, 28). 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:

Strain (λ) = Δ d/d(i)

d= diameter; d(i)= initial diameter

Stress (t) = λL/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 (25, 47). 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 (6, 14, 28). See **Figure 2** for representative stress-strain curve.

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) (25). To determine the boundaries of the elastin region of our stress strain curves, we calculated the roots of the second derivative of a 7^{th} 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 (25). 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. See **Figure 2** for representative stress-strain curve.

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187 ***Aortic Protein Expression***

188 Aortic expression of structural proteins collagen-I and α-elastin was determined in aortic
189 homogenates by standard Western blotting techniques and immunohistochemistry (IHC) in aortic
190 sections, as previously described (6, 11, 28). Aortic protein expression of inflammatory
191 cytokines was determined using a custom multiplex ELISA (Ciraplex, Aushon Biosystems,
192 Billerica, MA, USA), as previously described (27, 29).

193 Prior to Western blotting and cytokine multiplex, aortas were homogenized in radio-
194 immunoprecipitation assay lysis buffer and protein concentration determined using the Pierce
195 BCA assay kit (ThermoFisher Scientific, USA).

196 For Western blotting, 15 µg of aortic protein were loaded onto 4-12% polyacrylamide
197 gels and then transferred onto nitrocellulose membranes (Criterion System; Bio-Rad, Hercules,
198 CA, USA). Membranes were incubated (overnight at 4ºC) with primary antibodies: collagen-I
199 (1:1000, Millipore Corp.), α-elastin (1:200, Abcam, Inc., Cambridge, MA, USA), and
200 glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling, 1:1000, normalizer).
201 Proteins were visualized on a digital acquisition system (ChemiDoc-It, UVP, Upland, CA, USA)
202 using chemilluminescence with horseradish peroxidase-conjugated secondary antibodies
203 (Jackson ImmunoResearch, Westgrove, PA, USA) and ECL substrate (Pierce, Rockford, IL,
204 USA). Relative intensity was quantified using ImageJ software and normalized to GAPDH
205 intensity (obtained from the same blots after stripping) and then expressed as a ratio of the mean
206 intensity of the young control group.

For cytokine multiplex, 15 ug of aortic lysate were loaded into microplate wells and assay was performed according to manufacturer instructions. The multiplex plates were custom designed (custom Ciraplex, Aushon) for detection of the following murine pro-inflammatory cytokines: interleukins 1 beta, 6, and 10 (IL-1β, IL-6, and IL-10), and interferon gamma (IFN-γ). Images were captured using Cirascan imager (Aushon) and results were analyzed with Cirasoft software (Aushon). If levels of a given cytokine were undetectable (e.g., fell below the limit of detection of the assay), samples were excluded from the analysis.

For IHC, ~ 1 mm thoracic aortic segments were frozen in OCT compound in liquid nitrogen-cooled isopentane prior to sectioning. Aortic sections (7 μm) were fixed in acetone, washed in Tris buffer, and stained using the Dako EnVision+ System-HRP-DAB kit, as performed previously in our laboratory (11). Sections were incubated for 1 h at 4°C with primary antibodies for α-elastin (1:50, Abcam Inc.) or collagen-I (1:200, Millipore) and then incubated with the labelled polymer secondary for 30 minutes. Slides were dehydrated and cover-slipped after a 10-minute or 1-minute exposure to diaminobenzidine (elastin and collagen, respectively).

Stained aortic sections were imaged using a Nikon Eclipse TS100 photomicroscope under identical conditions. Quantification of the integrated density of the stain was performed using ImageJ software by a single investigator blinded to the group assignment of each sample. Collagen-I expression was assessed in the whole artery sections, comprising both the medial and adventitial layers, whereas elastin expression was assessed in the medial layer, the primary site of age-related changes in elastin expression (9, 10). Integrated density values from 4 sections were averaged to provide a single value for each protein per aorta, which are expressed relative to the mean of the young control group.

Statistical Analysis

All statistical analyses were performed using SPSS 23.0 software (Armonk, NY, USA). Data were first assessed for outliers and normality/homogeneity of variance. Between-group differences in morphological characteristics and aortic protein expression (Western blot, immunohistochemistry, multiplex ELISA) were determined using one-way analysis of variance. Between-group differences in elastic modulus (collagen and elastin regions) 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 and blood pressure were examined using a linear mixed model that also 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; (15)). Select morphological characteristics and blood pressure are shown in **Table 1**. Consistent with our previous study (15), 4 weeks of 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 reverses aortic stiffening in old mice

252 At baseline, aPWV was significantly higher in old compared to young mice and aPWV
253 was not significantly different from baseline to post-intervention in either young or old control
254 mice receiving normal drinking water (**Figure 1**). In contrast, 4 weeks of MitoQ treatment
255 significantly decreased aPWV in old mice to levels similar to young mice following the
256 intervention period. MitoQ treatment had no effect on aPWV in young mice. These results
257 indicate that 4 weeks of MitoQ treatment specifically reverses aortic stiffening in old mice.
258
259 ***Potential mechanisms underlying the de-stiffening effects of MitoQ treatment in old mice***
260 In our previous study employing MitoQ treatment in old mice (15), the same dose and
261 duration of treatment as used in the present study normalized the age-related elevation in aortic
262 whole-cell and mitochondria-specific superoxide production, indicating a profound antioxidant
263 effect of MitoQ in arteries. To investigate further how decreased levels of mtROS in aging
264 arteries may contribute to the de-stiffening effects of MitoQ, in the present study we investigated
265 key mechanisms that have been implicated downstream of mitochondrial oxidative stress in the
266 development of age-related arterial stiffening, namely changes in arterial structural proteins and
267 inflammation.
268
269 *Ex-vivo aortic stiffness—collagen- and elastin- mediated mechanical properties of aortic rings*
270 The elastic modulus of the collagen region of stress-strain curves was significantly
271 greater in old control versus young control mice (**Figure 3A**), whereas the elastic modulus of the
272 elastin region was significantly lower in old control compared to young control mice (**Figure**
273 **3B**), indicating an age-related increase in intrinsic arterial stiffness mediated by increased
274 collagen and reduced elastin. 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 attenuation of the age-related decline in elastin.

Aortic expression of structural proteins

Consistent with our intrinsic mechanical stiffness observations, aortic collagen protein expression was significantly greater (**Figure 4A and B**) and aortic elastin expression was lower (**Figure 4C and D,** p=0.074 and 0.086, respectively) in old control versus young control mice. MitoQ treatment did not affect aortic collagen content, such that collagen expression in old MitoQ-treated mice was not significantly different than that 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 levels in old MitoQ-treated mice were intermediate between (and not significantly different from) those 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 (9, 10)—elastin content in old MitoQ-treated mice was greater than that of old control mice (p=0.07).

Together with our observations of intrinsic mechanical properties, these results suggest that the reduction in *in vivo* aortic stiffening in old mice following MitoQ treatment was mediated not by effects on aortic collagen, but possibly by partial preservation of elastin.

Aortic inflammatory cytokine expression

Aortic expression of pro-inflammatory cytokines IL-6, IL-10, and IFN-γ (**Figure 5 A-C**) was significantly higher, and expression of IL-1β (**Figure 5D**) tended to be higher, in old compared to young control mice, consistent with previous investigations demonstrating elevated levels of arterial cytokines with aging and association with vascular dysfunction (3, 27, 29, 44). Cytokine levels were not affected by 4 weeks of MitoQ treatment ($p>0.05$ OMQ vs. OC for all cytokines), suggesting that the de-stiffening effects of MitoQ were not mediated by changes in these aortic cytokines. However, these results do not preclude the possibility that MitoQ treatment may have influenced other components of inflammatory signaling pathways.

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 aortic stiffness, assessed *in vivo* as aPWV. Our observation that MitoQ treatment decreases aortic stiffness in old mice extends previous work with general antioxidant compounds and adds to the evidence from transgenic and disease models that specifically implicates *mitochondrial* oxidative stress as a key contributor to aortic stiffening. A previous pre-clinical intervention study from our laboratory employing the general antioxidant compound TEMPOL established oxidative stress as a key mechanism underlying age-related aortic stiffening (12), and other strategies that decrease arterial oxidative stress also ameliorate arterial stiffness (11, 13, 14, 28, 49). Recent work with genetic and disease models indicates that mitochondria are a major source of the vascular oxidative stress contributing to arterial stiffness. Mice with genetic deletion of mitochondrial antioxidant enzyme SOD2, a model of excess mitochondrial oxidative stress, demonstrate exacerbation of age-related aortic stiffening (61), and progression of age-

related arterial stiffening is unaffected in mice with genetic deletion of cytosolic pro-oxidant NADPH oxidase (NOX1/2) but intact mitochondria-localized NADPH oxidase (NOX4) (55). Our finding here that *in vivo* treatment with the mitochondria-targeted antioxidant MitoQ in old mice decreases aortic stiffness provides further support for mitochondrial oxidative stress as a key mediator of arterial dysfunction with primary aging. Most importantly, our results extend previous observations from genetic and disease models (55) by demonstrating that a pharmacological intervention targeting excessive mtROS production reverses aortic stiffening in the setting of primary aging in mice, thus establishing an essential platform for translation to humans.

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 decreased whole cell and mitochondrial oxidative stress, are primarily mediated by decreases in arterial collagen content (9, 11, 12, 14, 37, 55), we observed that MitoQ treatment had no significant effect on the collagen region elastic modulus or aortic collagen expression but instead attenuated the age-related decline in aortic elastin region elastic modulus and tended to preserve elastin expression. Our finding of partial elastin preservation with MitoQ treatment is consistent with the observations that heterozygous SOD2 deficient mice, a model of excess mtROS, show marked exacerbation of age-associated declines in arterial elastin content (61), and that lifelong caloric restriction, a setting of lower mtROS (26), preserves arterial elastin content with aging (8). Collectively, our results suggest that decreasing mitochondrial oxidative stress may at least partially preserve elastin content in the aorta, contributing to lower levels of stiffness.

Future studies are warranted to elucidate the mechanisms by which decreased mitochondrial oxidative stress (via MitoQ treatment) may preserve aortic elastin content in aging. One possible link may be mtROS-mediated regulation of enzymes that govern elastin turnover, including matrix metalloproteinases (MMP; 36, 62)—changes in the activity of which are associated with arterial stiffening in both mouse models and human aging (32, 33, 58). For example, increased levels of MMP-2, a key enzyme involved in elastin degradation (9, 17, 59), accompany the loss of arterial elastin in heterozygous SOD2 knockout mice (61). Further, primary aging in preclinical models is associated with increased arterial MMP-2 expression (9, 59) and elevated aortic MMP-2 levels are also observed in human aging (33). Collectively, these previous studies suggest that age-related increases in mtROS may contribute to arterial elastin degradation via increased MMP-2 activity, and that targeting excess mtROS, e.g., via MitoQ treatment, may attenuate elastin degradation, preserving elastin content in large elastic arteries and contributing to lower levels of stiffness. Although our results do not support a role for MitoQ in decreasing total arterial collagen content, future studies could examine not only arterial content of this key structural protein, but also changes in collagen fiber orientation (17) and formation of cross-links among proteins, both of which have the potential to influence arterial stiffness (9, 23, 62).

It is also important to consider mechanisms other than preservation of aortic elastin content that may have contributed to the dramatic decrease in aortic 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 (17, 24, 62). 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 (15) 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., nitric oxide, regulation of structural protein turnover) mediators of large elastic artery stiffness *in vivo* (17, 32, 38, 60, 62).

Aortic inflammatory cytokine levels were significantly elevated in aortic tissue of old versus young mice, consistent with previous studies (27, 29, 44). Chronic low-grade arterial inflammation with aging, primarily mediated by NFκ-B activation, can be triggered by excessive oxidative stress—including that derived from mitochondria—in a reciprocally-reinforcing process that serves to impair arterial function (3, 29, 54). Although there is some evidence for a role of mtROS in mediating arterial inflammation and consequent dysfunction in atherosclerosis/disease models (31, 55), our observations in the present study do not support an anti-inflammatory role for MitoQ in reversing arterial stiffening in primary aging. Following 4 weeks of MitoQ treatment, there was no difference between old control and old MitoQ-treated aortic cytokine levels, despite the pronounced reversal of arterial stiffening in the latter. This suggests that the de-stiffening effects of MitoQ were mediated by a mechanism other than normalization of the aortic cytokines we assessed here. However, it remains possible that MitoQ treatment influenced other components of inflammatory signaling and future studies are warranted to investigate these possibilities.

Although the present study investigated the therapeutic efficacy of MitoQ in the setting of existing age-related aortic stiffness, it would also be of clinical relevance to determine whether targeting/decreasing mtROS earlier in life prior to the onset of aortic stiffening could

prevent or slow the progression of pathological aortic remodeling and consequent cardiovascular sequelae. Given that excess mtROS are implicated as a key factor in the pathogenesis of numerous age-related conditions, including vascular dysfunction (1, 4, 5), it is possible that limiting an age-related increase in mtROS via treatment in early or mid-life could prevent aortic stiffening. This possibility is supported by work from disease and senescence models indicating that mitochondria-targeted therapeutics initiated prior to or at the onset of experimental insult or injury can prevent development or slow progression of dysfunction (45, 51, 52). Because low, physiological levels of mtROS are critical for the maintenance of cellular homeostasis, any optimal long-term therapeutic strategy would likely need to maintain mtROS at physiological levels rather than eliminate them completely.

Conclusion

In conclusion, the present study demonstrates that late-life treatment with a mitochondria-targeted antioxidant, MitoQ, effectively reverses aortic stiffening in the setting of primary aging. Our results suggest that this effect is mediated at least partially by attenuation/reversal of the age-related reduction in aortic elastin content, but additional work is needed to conclusively determine the mechanism(s) underlying the de-stiffening effect of MitoQ. Importantly, these results indicate that mitochondria-targeted antioxidants may represent a novel, promising therapeutic strategy for decreasing aortic stiffness, and potentially decreasing the risk of multiple chronic age-associated conditions in humans.

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AUTHOR CONTRIBUTIONS

Conception and design of experiments: RGR, MPM, DRS

Performance of experiments/data collection: RGR, MLB, LMC, JSE

Data analysis: RGR, MLB, LMC, JSE

Data interpretation: RGR, MLB, LMC, JSE, MPM, DRS

Manuscript writing: RGR, DRS

Critical revision if manuscript: RGR, MLB, LMC, JSE, MPM, DRS

Final approval of manuscript: RGR, MLB, LMC, JSE, MPM, DRS

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DISCLOSURES

MPM is on the scientific advisory board of Antipodean Pharmaceuticals, Inc. All other authors declare that they have no conflicts of interest.

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	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 ± 28	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 Post: 101.4 ± 12.9	Pre: 101.3 ± 11.7 Post: 94.9 ± 5.0	Pre: 101.2 ± 6.7 Post: 98.2 ± 10.8	Pre: 93.5 ± 10.8 Post: 101.0 ± 4.4
Diastolic BP (mmHg)	Pre:73.3 ± 11.5 Post: 71.9 ± 11.3	Pre: 72.8 ± 11.0 Post: 67.0 ± 4.6	Pre: 73.1 ± 4.7 Post: 74.1 ± 9.3	Pre: 66.3 ± 4.2 Post: 71.9 ± 10.1

Table 1. General morphological characteristics and blood pressure

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 aortic 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. Representative stress-strain curve for determination of *ex-vivo* intrinsic mechanical stiffness of aortic rings.

Aortic rings were incrementally stretched until tissue failure, as described in the Methods section, and the tension (stress, kPa) corresponding to each stretch was plotted against strain (change in length relative to resting length) to generate a stress-strain curve. The elastic modulus of the region of the curve corresponding to collagen fiber stretching was determined as the slope of the line fit to the final 4 points on the curve prior to tissue failure (Collagen Region Elastic Modulus). The region of the curve corresponding to elastin fiber stretching was considered to lie between the very low-force region and the onset of collagen fiber engagement, which were identified as the first and second roots, respectively, of a 7^{th} order polynomial fit to the stress-strain curve (25). The elastic modulus of the elastin region of the curve was determined as the slope of the line fit between these boundaries (Elastin Region Elastic Modulus).

Figure 3. 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 4. MitoQ treatment attenuates the age-related reduction in aortic elastin expression but has no effect on aortic collagen expression.

A: Aortic collagen-I 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, comprising 4 continuous lanes, are presented below mean data. The collagen-I and GAPDH images represent the same segment of the same blot. Any adjustments to the images were limited to changes in brightness and contrast made using Image J to optimize visualization, performed uniformly on the entire image. n=6/group

* $p<0.05$ vs. YC

B: Aortic collagen-I expression assessed by immunohistochemistry in whole 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 (whole sections and enlargements of the same sections) are presented to the right of the mean data. n=7-11/group

* $p<0.05$ vs. YC

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, comprising 4 continuous lanes, are presented below mean data. The elastin and GAPDH images represent the same segment of the same blot. Any adjustments to the images were limited to changes in brightness and contrast made using Image J to optimize visualization, performed uniformly on the entire image. n=6/group

^ $p<0.074$ vs. YC

D: Aortic elastin expression assessed by immunohistochemistry in the medial layer of 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 (whole sections and enlargements of the same sections) are presented to the right of the mean data. n=8-11/group

^ $p=0.086$ vs. YC; ^^ $p=0.075$ vs. OC

Figure 5. MitoQ treatment does not affect the age-related increase in aortic inflammatory cytokines.

Expression of inflammatory cytokines **A)** interleukin-6 (IL-6, n=7-10/group), **B)** interleukin-10 (IL-10, n=7-9/group), **C)** interferon-gamma (IFN-γ, n=7-10/group), and **D)** interleukin-1 beta (IL-1β, n=4-10/group) in aortic homogenates from young and old control (YC and OC) and young and old MitoQ-treated (YMQ and OMQ) mice. Sample sizes reflect all aortic homogenates for which cytokine levels were detectable; samples were excluded if cytokine levels were undetectable/below the limit of quantification of the assay. Error bars represent SEM.

* $p<0.05$ vs. YC

^ $0.10>p>0.05$ vs YC ($p =0.08$, OC vs. YC; $p =0.06$, OMQ vs. YC).