

1 **Age-related endothelial dysfunction in human skeletal muscle feed arteries:**
2 **The role of free radicals derived from mitochondria in the vasculature**

3 Running Title: Mitochondria derived free radical in vasculature
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38 **NEW & NOTEWORTHY**

39 1. What is New?

40 • Free radicals from vascular mitochondria with advancing age play a critical role in attenuating
41 NO bioavailability and, subsequently, promote endothelial dysfunction in the skeletal feed
42 arteries (SMFAs) of the elderly.

43 • Mitochondria-targeted antioxidant, MitoQ, acutely restores SMFA endothelial function in the
44 old to that of the young.

45 2. What is Relevant?

46 • Scavenging free radicals from within the mitochondria of the vasculature with mitochondria-
47 targeted antioxidants reverses age-related vascular dysfunction which is a linked to
48 cardiovascular disease.

49 3. Summary

50 • Mitochondria-targeted antioxidants, such as MitoQ, may be a useful pharmacological therapy
51 in terms of counteracting the vascular dysfunction so often associated with advancing age and
52 cardiovascular disease (CVD).

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69 **ABSTRACT**

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71 This study sought to determine the role of free radicals derived from mitochondria in the
72 vasculature in the recognized age-related endothelial dysfunction of human skeletal muscle feed
73 arteries (SMFAs). A total of 44 SMFAs were studied, 18 from young (32 ± 6 yrs) subjects in
74 control conditions and 26 from old (75 ± 7 yrs) subjects with and without acute exposure to the
75 mitochondria-targeted antioxidant MitoQ and nitric oxide synthase (NOS) blockade. The
76 relative abundance of SMFA proteins from the electron transport chain (ETC), phosphorylated
77 (p-) to endothelial (e) NOS ratio, manganese superoxide dismutase (MnSOD), and the
78 mitochondria-derived superoxide (O_2^-) production were assessed. Endothelium-dependent and -
79 independent SMFA vasodilation was assessed in response to flow-induced shear stress,
80 acetylcholine (ACh), and sodium nitroprusside (SNP). The ETC proteins were lower in the old
81 and were not altered by MitoQ. MitoQ restored endothelium-dependent vasodilation in the old to
82 that of the young when stimulated by both flow (Young: 68 ± 5 ; Old: 25 ± 7 ; Old+MitoQ 65 ± 9 %)
83 and ACh (Young: 97 ± 4 ; Old: 59 ± 10 ; Old+MitoQ: 98 ± 5 %), but did not alter, the initially
84 uncompromised, endothelium-independent vasodilation (SNP). Compared to the young, MitoQ
85 in the old attenuated the initially elevated mitochondria-derived O_2^- production and increased the
86 initially attenuated level of MnSOD. Furthermore, MitoQ increased the ratio of p-eNOS/NOS
87 and the restoration of endothelium-dependent vasodilation in the old by MitoQ was ablated by
88 NOS blockade. Thus, free radicals derived from mitochondria in the vasculature of the elderly
89 appear to play a critical role in attenuating NO bioavailability and, subsequently, endothelial
90 dysfunction with advancing age. (Words 253)

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95 **ABBREVIATIONS LIST**

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97 SMFAs, skeletal muscle feed arteries; NOS, nitric oxide synthase; ETC, electron transport chain;

98 MnSOD, manganese superoxide dismutase; O_2^- , superoxide; ACh, acetylcholine; SNP, sodium

99 nitroprusside; NO, nitric oxide; TPP, triphenylphosphonium; L-NMMA, N^o-nitro-L-arginine

100 methyl ester; PSS, physiological saline solution; ONOO⁻, peroxynitrite; CVD, cardiovascular

101 disease

102

103 **INTRODUCTION**

104

105 With advancing age, blood flow to skeletal muscle is often diminished (24, 31), which, at least in

106 part, is likely a consequence of attenuated endothelial function in the skeletal muscle resistance

107 vasculature (9, 33, 34). However, the specific mechanism(s) responsible for the age-related

108 attenuation of skeletal muscle blood flow is currently not well understood. The study of human

109 SMFAs is highly germane to better understanding the vascular biology of aging, as it affords the

110 opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood

111 flow, also have regulatory potential (18). Indeed, our group has recently documented that the

112 vasodilatory function of SMFAs obtained from elderly human subjects was markedly attenuated

113 and this functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS

114 protein levels (29). Although attenuated NO bioavailability with advancing age may depend on

115 multiple factors that regulate NO production and degradation, free radicals, principally O_2^- (2, 14,

116 21), likely play an important role by reacting rapidly with NO, thereby decreasing NO

117 bioavailability (21, 37). Currently, the exact source of the free radicals that appear to attenuate

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

119 Mitochondria play a critical role in cellular function in both health and disease, but are also an

120 important and major source of free radicals (22, 35). Interestingly, although mitochondrial

121 content is relatively low in vascular endothelial cells and smooth muscle (2-5% of cell volume)
122 compared to physically active skeletal muscle and cardiac myocytes (5-35 % of cell volume)
123 (11), previous studies have revealed a strong correlation between mitochondria-derived oxidative
124 stress and endothelial dysfunction (2, 8, 35). Interestingly, our group recently documented that
125 exercise training induces an increase in vascular mitochondrial respiratory capacity, evidence of
126 improved redox balance, and elevated basal NO bioavailability (30). These data suggest that age-
127 and disease-related alterations in arterial function may be directly affected by the function, and
128 subsequent free radical production, of mitochondria in the vasculature. Therefore, strategies to
129 constrain mitochondria-derived free radical levels to within typical physiological levels may
130 prove useful in attenuating the development of endothelial dysfunction with age.

131 The first line of defense against free radicals is both endogenous and exogenous antioxidants.
132 However, to date, antioxidant supplementation (e.g. Vitamin C) has not proven effective at
133 specifically decreasing mitochondria-derived free radical production (1, 19). Of note, as
134 mitochondria are negatively charged, the incorporation of a lipophilic cation, such as
135 triphenylphosphonium (TPP), to a potent antioxidant, such as the active ubiquinol moiety of
136 Coenzyme Q10, enables the selective and extensive accumulation of the antioxidant within the
137 mitochondria (26, 27). Utilizing this approach, a commercially available mitochondria-targeted
138 antioxidant, MitoQ (MitoQ Limited, Auckland, NZ), has been synthesized to yield a thousand-
139 fold greater concentration within the mitochondria than untargeted antioxidants, which distribute
140 throughout the cell (26, 27). The use of MitoQ to specifically treat age-related endothelial
141 function is supported by a recent, elegant and comprehensive, study by Gioscia-Ryan et al., (15)
142 who reported that this mitochondria-targeted antioxidant attenuated endothelial dysfunction in

143 older mice. Nevertheless, age-related vascular mitochondrial free radical production and
144 endothelial dysfunction in humans has yet to be examined.

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

150 **METHODS**

151 **Subjects and general procedures:** A total of 44 SMFAs were obtained from young and old
152 subjects, from the axillary and inguinal regions, during melanoma-related surgeries. From these
153 SMFAs, endothelial-dependent and -independent vascular function was assessed in 10 young
154 subjects, while 16 old subjects were assessed with and without MitoQ. A subset of these vessels
155 (n = 8 young and 8 old subjects) were assessed for mitochondria-specific O_2^- production.
156 Endothelial-dependent vascular function was assessed in the SMFAs from the remaining 8 young
157 subjects, while the remaining 10 old subjects were assessed with and without MitoQ and N^o -
158 nitro-L-arginine methyl ester (L-NMMA). Unused segments of these vessels (n = 8 young and
159 10 old subjects) were used for immunoblotting. It should be noted that, although all subjects
160 were free from cancer and chemotherapy, there were no other specific exclusion criteria for this
161 study. However, all medical conditions and medications were noted. All protocols were
162 approved by the Institutional Review Boards of the University of Utah and Salt Lake City
163 Veteran's Affairs Medical Center (VAMC), carried out in accordance with the Declaration of
164 Helsinki, and written informed consent was obtained from all subjects prior to surgery.

165 **Vessel harvest and preparation:** SMFAs (outer diameter ~500 μm , length 1-2 cm) from the
166 axillary (e.g. serratus anterior or latissimus dorsi muscles) and inguinal (e.g. hip adductors or
167 quadriceps femoris muscles) regions, obtained during sentinel node biopsy for melanoma surgery
168 at the Huntsman Cancer Hospital and the Salt Lake City VAMC, were studied. Patients were
169 anaesthetized using a general protocol: propofol, fentanyl, benzodiazepines, and succinylcholine
170 (28). SMFAs were harvested during dissection to locate sentinel lymph nodes, for clinical
171 analysis, and were identified and classified based upon being a vascular inlet into a muscle bed,
172 structure, coloration, and pulsatile bleed pattern (17). SMFAs were ligated, excised, and
173 immediately placed in iced normal physiological saline solution (PSS) before being transferred
174 to the laboratory within 15 min of harvesting (29).

175 **MitoQ treatment and vessel function protocols:** Initially, perivascular adipose and/or
176 connective tissue around the SMFAs was removed under a dissecting microscope (SZX10;
177 Olympus, Center Valley, PA, USA) in cold (4 $^{\circ}\text{C}$) PSS containing (mM): 145.0 NaCl, 4.7 KCL,
178 2.0 CaCl_2 , 1.17 MgSO_4 , 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS buffer and 1 g (100
179 mL^{-1} BSA at pH 7.4. SMFA function was assessed in pressure myography organ baths (110p;
180 DMT Systems, Aarhus, Denmark) (29). The arteries were cannulated at both ends with
181 micropipette tips and then pre-incubated for 30 min within the bath in either PSS, the control
182 condition, or MitoQ mesylate (10 μM). After the pre-incubation period, the vessel outer
183 diameters were recorded using an inverted microscope with a video camera (TS100; Nikon
184 Eclipse, Melville, NY, USA), with data streamed in real time to edge detection software (DMT
185 VAS v 0.2.0), monitored at a sampling rate of 1 kHz. Fluid leak was detected by pressurizing the
186 vessel to an intraluminal pressure set of 60 mmHg, closing the cannulas to the fluid reservoirs,
187 and assessing any change in vessel diameter. Arteries, free from leaks were then warmed to 37 $^{\circ}\text{C}$,

188 allowed to develop spontaneous tone for a 30 min equilibration period, and then vasodilatory
189 function was assessed (29).

190 **Vasodilation assessments:** Vasodilatory dose response curves (%) were assessed for three
191 stimuli: First, to assess the endothelium-dependent vasodilatory response to flow-induced shear
192 stress, intraluminal flow was developed. This was achieved by altering the heights of the
193 independent fluid reservoirs, contiguous with both cannulated ends of the SMFAs, in equal and
194 opposite directions so that a pressure difference was developed across the vessel without altering
195 mean intraluminal pressure. Three pressure differences of 15, 30, and 45 mmHg, which yielded
196 an approximate flow rate of 15, 30 and 45 $\mu\text{L}/\text{min}$, were utilized for the flow experiments. Second,
197 to assess endothelium-dependent vasodilation pharmacologically, an ACh dose response curve
198 (ACh, 10^{-7} to 10^{-3} M) was performed following pre-constriction with phenylephrine (PE) (10^{-6} to
199 10^{-4} M) to ~ 70 % of the maximum PE response. Third, to assess endothelium-independent
200 vasodilation, a SNP dose response curve was performed (10^{-9} to 10^{-4} M) following pre-
201 constriction with PE (10^{-6} to 10^{-4} M) to ~ 70 % of the maximum PE response.

202 **Mitochondria-specific O_2^- measurements:** Mitochondria-specific O_2^- measurements were
203 performed with EPR spectroscopy on the initially frozen SMFA segments using an EMX X-band
204 spectrometer (Bruker, MA). Briefly, the segment of the frozen SMFA was placed into a micro
205 centrifuge tube containing 150 μL of the mitochondria-specific O_2^- spin trap mitoTempo-H
206 (Enzo Life Sciences San Diego, CA) (1-hydroxy-4 [2-(triphenylphosphino) – acetamido] -
207 2,2,6,6-tetramethylpiperidine) (0.5 mmol/L) and incubated for 60 minutes at 37 $^\circ\text{C}$, facilitating
208 the “thaw and trap” approach (10, 32). The samples were then placed on ice and 50 μL of the
209 solution was loaded into a capillary tube for EPR spectroscopy analysis. The EPR spectroscopy

210 scan was run with a center field at approximately $g = 2.004$ and the area under the curve of the
211 spectra was calculated by double integration (29).

212 **Percent vasodilation calculations:** Percent vasodilation was used for data expression to account
213 for baseline differences in vessel diameter, and calculated using the following equation:

$$214 \quad (DT-D_p/D_i-D_p) \times 100$$

215 Where DT is the recorded diameter at a given time point, D_p is the diameter recorded after the
216 addition of the vasoactive agent (i.e. pre-constriction diameter), and D_i is the diameter recorded
217 immediately before the addition of the vasoactive agent (initial diameter).

218 **Immunoblotting:** The relative abundance of proteins for the ETC complexes, p- and eNOS, and
219 MnSOD were determined in SMFAs using Western blot analysis. Briefly, SMFAs were
220 homogenized in lysis buffer, supplemented with a protease/phosphate inhibitor cocktail (10 μ M
221 sodium fluoride and 1 mM phenyl methyl sulfonyl fluoride (PMSF)) (Santa Cruz Biotech, Santa
222 Cruz, CA). Protein concentration was determined using the Bradford technique. 50 μ g of
223 homogenate was separated by polyacrylamide gel electrophoresis, transferred onto a
224 nitrocellulose membrane, and incubated with primary and secondary antibodies directed against
225 the proteins of interest. Membranes were imaged on a ChemiDoc XRS (Bio-Rad, Hercules, CA)
226 and quantified with Image Lab software (Bio-Rad). The specific antibodies used to detect SMFA
227 proteins included: Total OXPHOS Human Western Blot Antibody Cocktail (ab110411, Abcam,
228 Cambridge, MA), total eNOS (610296, BD Transduction, San Jose, CA), p-eNOS at Ser1177
229 (9570, Cell Signaling, Boston, MA), and superoxide dismutase 2 (SOD2) (SC-515068, Santa
230 Cruz Biotech, Santa Cruz, CA). The abundance of each protein was normalized to beta-actin
231 (ab8227, Abcam, Cambridge, MA), which served as a loading control.

232 ***Statistical Analyses:*** The statistical analyses were performed using GraphPad Prism 7 Software
233 (La Jolla, CA). Two-way repeated measures ANOVA was used to assess changes in vessel
234 diameter with and without MitoQ in response to flow, ACh, and SNP. Two-way repeated
235 measures ANOVA were used to assess changes in vessel diameter with and without MitoQ and
236 with and without L-NMMA in response to flow and ACh. When necessary, a Tukey's post hoc
237 test was used to identify significant differences. For all other comparisons, one-way ANOVA
238 was used to assess the group and, if necessary, a Tukey's post hoc test was used to identify the
239 significant differences. For all analyses, a p-value of < 0.05 was considered significantly
240 different. All data are expressed as mean \pm SEM.

241 **RESULTS**

242 **Subject characteristics:** From the 44 SMFAs that were harvested, 18 were from young subjects
243 (33±2 yrs) and 26 were from old subjects (72±5 yrs). The subject characteristics, obtained from
244 preoperative examination of medical records, are presented in Table 1. Note that users of cancer-
245 related medications were excluded from the study. Also, it should be noted that all blood
246 chemistry and complete blood count results (Table1) were within normal ranges, suggesting that
247 the subjects who participated in this study were relatively healthy.

248 **Vessel characteristics:** SMFAs were harvested from either the inguinal (n=23) or axial (n=21)
249 regions from either males (n=25) or females (n=19). In agreement with our previous
250 observations, vessel function was not different as a consequence of anatomic origin or sex.
251 Immunoblotting, to assess the relative abundance of proteins in the ETC, revealed that the
252 majority of the mitochondrial respiratory complexes, with the exception of Complex V, were
253 significantly attenuated in the SMFAs of the old compared to the young (Figure 1). MitoQ did
254 not alter this attenuation of the mitochondrial respiratory complexes in the old (Figure 1). Basal,
255 unpressurized, outer diameter of the SMFAs was not statistically different in the young, old, and
256 old with MitoQ (Young: 510 ± 12 μm ; Old: 514 ± 15 μm ; Old+MitoQ: 515 ± 10 μm).
257 Additionally, maximal outer diameter of the SMFAs, achieved by Ca^{2+} free NPSS incubation,
258 was not statistically different in the young, old, and old with MitoQ (Young: 758 ± 19 μm ; Old:
259 752 ± 14 μm ; Old+MitoQ: 750 ± 15 μm).

260 **The vasodilatory response to flow, ACh, and SNP and the impact of MitoQ in the old:** The
261 PE-induced pre-constriction of the SMFAs prior to the flow stimulus was similar between groups
262 (Young: 69 ± 4 %, Old: 67 ± 5 %, Old+MitoQ: 68 ± 5, $P > 0.05$). The greatest vasodilation in

263 response to the intraluminal flow of 45 ± 3 ul/min was significantly attenuated in the old
264 compared to the young (Young: 68 ± 5 ; Old: $25 \pm 7\%$, $P < 0.05$) (Figure 2A). However, the
265 vasodilatory response to flow in the old was restored to that of the young by MitoQ (Old+MitoQ:
266 $65 \pm 9\%$) (Figure 2A). This effect of MitoQ in the old was also evident at the lower intraluminal
267 flow rates of 15 ± 2 and 30 ± 4 μ l/min (Figure 2A).

268 The PE-induced pre-constriction of the SMFAs prior to the ACh and SNP dose response
269 curves were similar between groups (Young: $69 \pm 4\%$; Old Control: $68 \pm 5\%$; Old + MitoQ 69
270 $\pm 5\%$, $P > 0.05$). The greatest vasodilation in response to the highest dose of ACh (10^{-3} M) was
271 significantly attenuated in the old compared to the young ACh (Young: $97 \pm 4\%$; Old: $59 \pm 10\%$,
272 $P < 0.05$) (Figure 2B). However, the vasodilatory response to ACh in the old was restored to that
273 of the young by MitoQ (Old+MitoQ: $98 \pm 5\%$) (Figure 2B). This effect of MitoQ in the old was
274 clearly evident across the whole ACh dose response curve (Figure 2B). In contrast, endothelial-
275 independent vasodilatory function, the vasodilatory response to the highest dose of SNP (10^{-4} M)
276 (Young: $97 \pm 4\%$, Old: $100 \pm 11\%$; Old+MitoQ: $98 \pm 4\%$, $P > 0.05$) and across the whole dose
277 response curve, was similar among the young, old, and old with MitoQ (Figures 2C).

278 **Levels of mitochondria-specific O_2^- and MnSOD and the impact of MitoQ in the old:** The
279 baseline EPR spectroscopy signal for the mitoTempo-H adduct in the SMFAs, an index of
280 mitochondria-specific O_2^- production, was greater in the old compared to the young (Young: 1.7
281 ± 0.2 ; Old: 6 ± 1.8 ; AUC/mg, $P < 0.05$) (Figures 3A). However, MitoQ significantly lowered
282 SMFA O_2^- production in the old, such that the old were similar to the young (Old+MitoQ: $1.95 \pm$
283 0.7 ; AUC/mg) (Figure 3A). In terms of antioxidant status, immunoblotting revealed that baseline
284 MnSOD protein content was significantly attenuated in the old compared to the young (Young:
285 100 ± 18 ; Old: 38 ± 17 AUC, $P < 0.05$) (Figure 3B). However, incubation with MitoQ

286 significantly increased the MnSOD protein content of the old (Old+MitoQ: 78 ± 15 AUC)
287 (Figure 3B).

288 **The role of NO bioavailability and the impact of MitoQ in the old:** Immunoblotting revealed
289 that the extent of eNOS phosphorylation, measured as the p-eNOS/eNOS ratio on the Western
290 blots, was significantly lower in the old compared to the young (Young: 100 ± 16 ; Old: 35 ± 18 ;
291 AUC $P < 0.05$). However, MitoQ enhanced the extent of eNOS phosphorylation in the old
292 (Old+MitoQ: 59 ± 18 AUC) (Figure 4). SMFA vasodilation, in response to both flow and
293 increasing doses of ACh, again revealed attenuated endothelial-dependent vasodilation in the old
294 which could be restored acutely by MitoQ (Figure 5A and B). However, the impact of the MitoQ
295 was negated by NOS blockade (Figure 5A and B). Furthermore, in the presence of L-NMMA the
296 vasodilatory response to both flow and ACh with and without MitoQ was attenuated to a level
297 that was significantly lower than the initial dose response in the old (Figure 5A and B).

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299

300 **DISCUSSION**

301 This study sought to determine the role of free radicals derived from mitochondria in the
302 vasculature in the age-related endothelial dysfunction documented in human SMFAs. The main
303 hypothesis tested by this investigation was that free radicals derived from aging vascular
304 mitochondria play a critical role in attenuating NO bioavailability and, subsequently, promote
305 endothelial dysfunction in the elderly. The current findings strongly support this postulate and, of
306 importance, translate previous findings in an animal model to humans. Specifically, despite the
307 observation that the ETC proteins were lower in the old, and this was not altered by MitoQ, this
308 mitochondria-targeted antioxidant acutely restored SMFA endothelium-dependent vasodilation,
309 in response to both flow and ACh, to that of the young. Additionally, MitoQ attenuated
310 mitochondria-derived O_2^- production, likely sparing MnSOD, which resulted in an increase in
311 MnSOD levels. Furthermore, in the old, the restoration of SMFA endothelium-dependent
312 vasodilation by MitoQ was ablated by NOS blockade, and MitoQ increased the extent of eNOS
313 phosphorylation. Thus, augmented mitochondrial free radical production in the SMFAs of the
314 elderly appears to play a critical role in attenuating NO bioavailability and, subsequently,
315 promoting endothelial dysfunction with advancing age.

316 **Vascular aging, SMFAs, free radicals, and NO bioavailability:**

317 In terms of the vascular biology of aging, the study of human SMFAs is pertinent, as it affords
318 the opportunity to examine endothelial function in vessels that, in terms of skeletal muscle blood
319 flow, also have regulatory potential (18). In fact, our group recently documented that the
320 endothelial function of SMFAs attained from the elderly was markedly attenuated and this
321 functional decline was associated with a decrease in the ratio of p-eNOS to total eNOS protein

322 level, emphasizing the likely role of attenuated NO bioavailability (29). Here, the findings of this
323 previous work were confirmed with further evidence that aging similarly attenuates both flow-
324 and ACh-mediated vasodilation in SMFAs (Figure 2A and B), each indicators of endothelium-
325 dependent vasodilation. The current findings further suggest that this limited vasodilatory
326 capacity with advancing age is, at least in part, due to attenuated NO bioavailability, as again
327 evidenced by a decrease in the ratio of p-eNOS to total eNOS protein expression in the SMFAs
328 from the old (29) (Figure 4). Attenuated NO bioavailability with advancing age depends on
329 multiple factors that regulate NO production and degradation, with a key role being played by
330 free radicals. For example, O_2^- decreases NO bioavailability (2, 14, 21) by rapidly reacting with
331 NO to form peroxynitrite ($ONOO^-$), but then, in turn, $ONOO^-$ may oxidise the essential co-factor
332 for eNOS, tetrahydrobiopterin, resulting in O_2^- production, rather than NO, by eNOS (21, 37).
333 This redox imbalance likely plays an important role in the age-related fall in NO bioavailability,
334 supported in this study by the greater mitochondria-derived O_2^- production and reciprocally
335 attenuated MnSOD levels in the old SMFAs (Figure 3A and B). Indeed, there is accumulating
336 evidence that increased free radical production leads to endothelial dysfunction with advancing
337 age both in animals and humans, and that the resultant oxidative stress promotes vascular disease
338 (7, 25, 39).

339 **MitoQ, age-related vascular dysfunction, and NO bioavailability:**

340 The acute 1 hr incubation of the SMFAs from the old with MitoQ effectively reversed the age-
341 related vascular dysfunction (Figure 2A and B). Several lines of evidence from this study suggest
342 that this restoration of vascular function in the old SMFAs was NO mediated. First, MitoQ
343 greatly attenuated mitochondrial O_2^- production to more closely resemble that of the young
344 (Figure 3A), a change that would likely result in an increase in NO bioavailability. Again, it is

345 interesting to note that this fall in O_2^- production was accompanied by an increase in MnSOD
346 (Figure 3B). This makes intuitive sense and suggests a MitoQ-induced sparing of this
347 endogenous antioxidant that targets O_2^- and is found predominantly within the mitochondria.
348 Second, MitoQ significantly increased the attenuated ratio of p-eNOS to total eNOS protein
349 expression in the SMFAs from the old (Figure 4), indicative of rescuing the activity of this NO
350 producing pathway. Third, the reversal of the age-related vascular dysfunction achieved by
351 MitoQ during both the flow and ACh dose response curves was ablated by NOS blockade,
352 confirming a role for NOS in the MitoQ-induced response. Furthermore, the flow and ACh
353 responses with and without MitoQ, in combination with NOS blockade, were significantly
354 attenuated compared to the flow and ACh assessments in the old SMFAs. Overall, this indicates
355 that NO still plays a role in the response of the old vessels, but, more importantly, that MitoQ
356 was ineffectual when NOS was blocked, implying an NO-mediated mechanism of action (Figure
357 2A and B). Although performed in stroke-prone hypertensive rats, the conclusion by Graham et
358 al. (16) that MitoQ supplementation, initiated prior to the establishment of cardiovascular disease
359 (CVD) in young animals, prevented the development of endothelial dysfunction by maintaining
360 NO bioavailability, is in agreement with the premise of the current findings.

361 **Vascular aging, SMFAs, blood flow, and oxygen transport:**

362 It is widely accepted that aging is commonly associated with impaired blood flow, and
363 subsequently oxygen delivery, to skeletal muscle during dynamic exercise and that this is likely
364 caused by a combination of compromised cardiac output (17, 23) and attenuated peripheral
365 vascular conductance with age (23, 25). In terms of the skeletal muscle vasculature, in rodent
366 studies, the rate of endothelium-dependent vasodilation in the skeletal muscle arterioles, which
367 are downstream from the SMFAs, and microcirculatory blood flow was attenuated in old

368 compared to young animals (3, 4), subsequently impairing oxygen delivery to the contracting
369 muscles. In humans, our group recently provided evidence supporting the contention that human
370 SMFAs, the inlets to the muscle bed upstream of the arterioles, regulate vascular resistance, and
371 therefore skeletal muscle perfusion, in response to shear stress and pharmacological vasodilators
372 (17, 29). Furthermore, our group has also demonstrated that SMFAs from older humans exhibit
373 an attenuated magnitude of endothelium-dependent vasodilation and delayed vasodilation
374 kinetics in response to shear stress and ACh (29). In agreement with these prior results, the
375 current findings confirm that the endothelium-dependent vasodilatory capacity of SMFAs,
376 assessed by flow-induced shear stress and the response to ACh, is clearly attenuated with
377 advancing age (Figure 2A and B). This attenuated SMFA vasodilation with aging is likely one of
378 the mechanisms responsible for the age-related decline in blood flow and oxygen transport to
379 active skeletal muscle during physical activity in the elderly. In light of the current positive
380 findings with MitoQ and the positive impact on age-related vascular function, additional studies
381 examining the effect of mitochondria-targeted antioxidants on skeletal muscle blood flow during
382 exercise in the elderly are warranted.

383 **Mitochondrial health, vascular aging, and MitoQ**

384 As the major energy producers for most physiologic processes, well-functioning, healthy,
385 mitochondria are essential for both systemic and cellular homeostasis. However, in addition to a
386 central role in energy production, mitochondria seem to be important in terms of molecular
387 signaling and cellular secretion in the vasculature and this is mediated, at least to some extent, by
388 free radicals (8, 11). Indeed, free radicals, produced at numerous sites within the mitochondria,
389 including Complexes I, II, and III of the ETC, play a critical role in these processes. For example,
390 it has been documented that mitochondria located in the endothelial cytoskeleton of arterioles, in

391 the human myocardium, produce free radicals in response to shear stress induced cell
392 deformation, which are critical for flow-mediated dilation (20). Conversely, several recent
393 studies have also revealed that mitochondria-derived free radicals in the vasculature play a
394 critical role in peripheral vascular dysfunction with advancing age (15, 36, 38). Interestingly, and
395 along these lines, both hyperglycemia and elevated triglycerides, recognized as inducers of
396 endothelial dysfunction and atherosclerosis, increase mitochondria-derived free radicals and alter
397 mitochondrial dynamics in vascular endothelial cells. This vascular dysfunction can be reversed
398 by normalizing the blood sugar and lipid load, removing the mitochondrial stimulus (6).
399 Furthermore, and perhaps somewhat ironically, in terms of mitochondrial health, mitochondria-
400 derived free radicals lower the abundance of MnSOD, which resides in the mitochondrial matrix,
401 and negatively impacts mitochondrial biogenesis and mitochondrial content (15).

402 The initial age-related findings from this study support the link between attenuated vascular
403 function with advancing age (Figure 2A and B) and compromised vascular mitochondrial health,
404 as evidenced by the greater O_2^- production (Figure 3A), lower levels of MnSOD (Figure 3B), and
405 the attenuation of the ETC complexes (Figure 1) in the SMFAs from the old. Interestingly, in
406 addition to restoring endothelial function in the SMFAs from the old (Figure 2A and B), the
407 acute 1 hour incubation with MitoQ both decreased mitochondrial O_2^- production (Figure 3A)
408 and restored mitochondrial antioxidant capacity (MnSOD) (Figure 3B). However, MitoQ did not
409 impact the relative abundance of ETC complex proteins (Figure 1). This is of particular
410 relevance in light of recent studies that have suggested aging is associated with attenuated
411 mitochondrial respiratory complexes (12) and that elevated mitochondria-derived free radical
412 production damages the mitochondrial DNA that encodes the ETC complexes (5, 13). This
413 damage, predominantly at complex I, appears to directly affect electron transport and disrupts the

414 whole mitochondrial respiratory cycle (5, 13). In the current study, although perhaps not
415 surprising, due to the relatively short time course of the MitoQ exposure, the lack of effect on the
416 significantly attenuated ETC complex protein expression is an important observation.
417 Specifically, this documents that the positive impact of MitoQ on vessel function and
418 mitochondrial free radical production is not dependent upon more long-term changes in the
419 relative abundance of the mitochondrial complexes.

420 **Conclusion**

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

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435 **Competing interests**

436 M. P. M is on the scientific advisory board of Antipodean Pharmaceuticals, Inc. All other authors
437 declare that they have no competing interests.

438 **Author contributions**

439 S.-Y.P., O.S.K, and R.S.R. designed and wrote the paper; O.S.K and S.-Y.P. performed
440 experiments and analyzed data; R.H.I.A. and J.R.H. provided SMFAs; M.P.M. provided MitoQ
441 and contributed to the revision of the article. All authors have approved the final version of the
442 manuscript, agree to be accountable for all aspects of the work and quality for authorship.

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455 **FIGURE LEGENDS**

456 **Figure 1. The relative abundance of skeletal muscle feed artery proteins from the electron**
457 **transport chain (ETC) of young subjects and old subjects with and without MitoQ.** The
458 ETC protein expression was normalized by β -actin protein expression. Data are expressed as
459 mean \pm SE. n = 10 young and 16 old subjects. * Significantly different from young, $P < 0.05$.

460 **Figure 2. The vasodilatory dose response curves of skeletal muscle feed arteries from young**
461 **subjects and old subjects with and without MitoQ evoked by flow, acetylcholine (ACh), and**
462 **sodium nitropruside (SNP).** Data are expressed as mean \pm SE. n = 10 young and 16 old
463 subjects. * Significantly different from old, $P < 0.05$.

464 **Figure 3. Mitochondria-specific superoxide production and manganese superoxide dismutase**
465 **(MnSOD) protein expression in skeletal muscle feed arteries of young subjects and old**
466 **subjects with and without MitoQ.** Superoxide levels were assessed utilizing the mitochondrial-
467 specific superoxide spin trap mitoTempo-H and electron paramagnetic resonance (EPR)
468 spectroscopy. The EPR signal was expressed as the area under the curve (AUC) in arbitrary units
469 and representative spectra are inlayed. The MnSOD protein expression was normalized by β -
470 actin protein expression. Data are expressed as mean \pm SE. n = 8 young and 8 old subjects for
471 EPR and n = 8 young and 10 old subjects for immunoblotting. MnSOD expression of young, old,
472 old + MitoQ. * Significantly different from young and old+MitoQ, $P < 0.05$; † Significantly
473 different from young, $P < 0.05$.

474 **Figure 4. The relative abundance of proteins for endothelial NOS (eNOS) and**
475 **phosphorylated (p-) eNOS at Ser1177 from skeletal muscle feed arteries of young subjects**
476 **and old subjects with and without MitoQ.** Data are expressed as mean \pm SE. n = 8 young and

477 10 old subjects. * Significantly different from young and old+MitoQ, $P < 0.05$; † Significantly
478 different from young, $P < 0.05$.

479 **Figure 5. The vasodilatory dose response curves of skeletal muscle feed arteries from young**
480 **subjects and old subjects both with and without MitoQ and with and without nitric oxide**
481 **synthase blockade (L-NMMA) evoked by both flow and acetylcholine (ACh). Data are**
482 **expressed as mean \pm SE. n = 8 young and 10 old subjects. * Significantly different from young**
483 **and old+MitoQ, $P < 0.05$; † Significantly different from all other groups and conditions, $P < 0.05$.**

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Table 1. Subject characteristics.

	Young (n=18)	Old (n=26)
Age (year)	32±6	75±7 *
Sex (male/female, n)	10/8	15/11
Height (cm)	175±15	165±12
Body mass (kg)	74±13	81±10
BMI (kg m ⁻²)	21±7	27±7
Systolic blood pressure (mmHg)	116±7	126±9
Diastolic blood pressure (mmHg)	78±5	81±9
Glucose (mg dl ⁻¹)	110.8±9.2	108±5.2
Blood urea nitrogen (mg dl ⁻¹)	17.4±5.0	16.8±6.4
Creatinine (mg dl ⁻¹)	0.9±0.7	1±0.9
Albumin (g dl ⁻¹)	4.2±0.6	4.2±0.7
Lactate dehydrogenase (U L ⁻¹)	505.4±40.1	503±47.3
Hemoglobin (g dl ⁻¹)	15.5±1.2	14.3±1.5
White blood Cells (thousands per microliter, K ul ⁻¹)	4.9±2.1	7.7±1.4
Red blood Cells (millions per microliter, M ul ⁻¹)	5.2±1.3	4.8±1.5
Platelets (K ul ⁻¹)	255.9±21.1	240±27.2
Hematocrit (%)	41.4±3.1	40±5
Lymphocytes (%)	34.3±3.3	33±8.5
Monocytes (%)	8.6±1.6	8.1±2.5
Medications (Users/n)		
Diuretics	0/18	2/26
Angiotensin- converting enzyme inhibitors	0/18	2/26
Diabetic drugs	0/18	3/26
Statins	0/18	2/26

Data are expressed as mean ± SE or number of subjects (of the total number; *n*).

*Significantly different from young subjects, *P*<0.05

Figure 1.

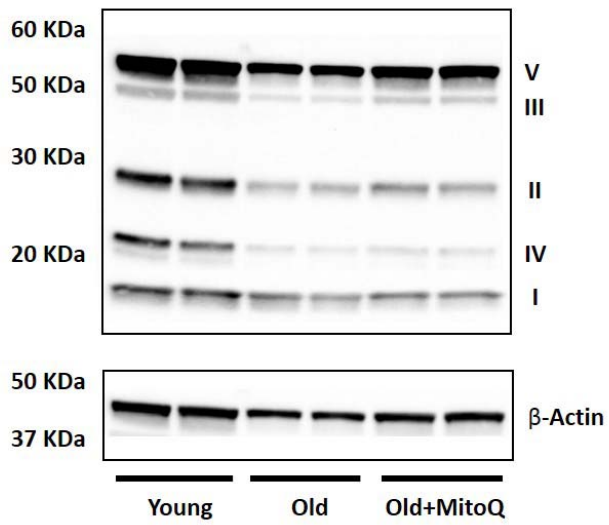
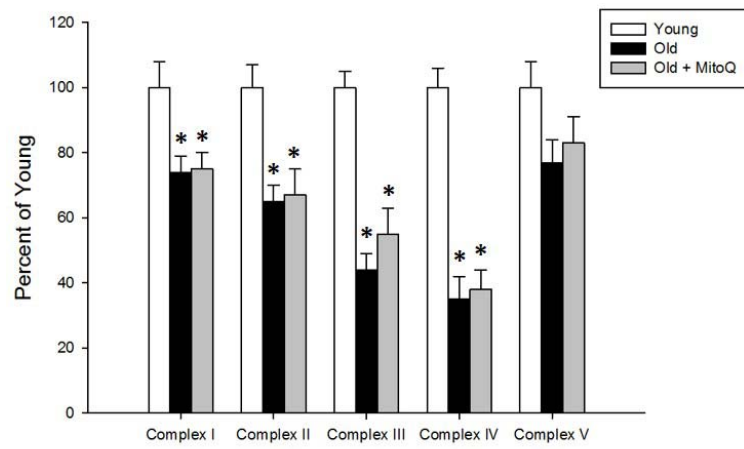


Figure 2.

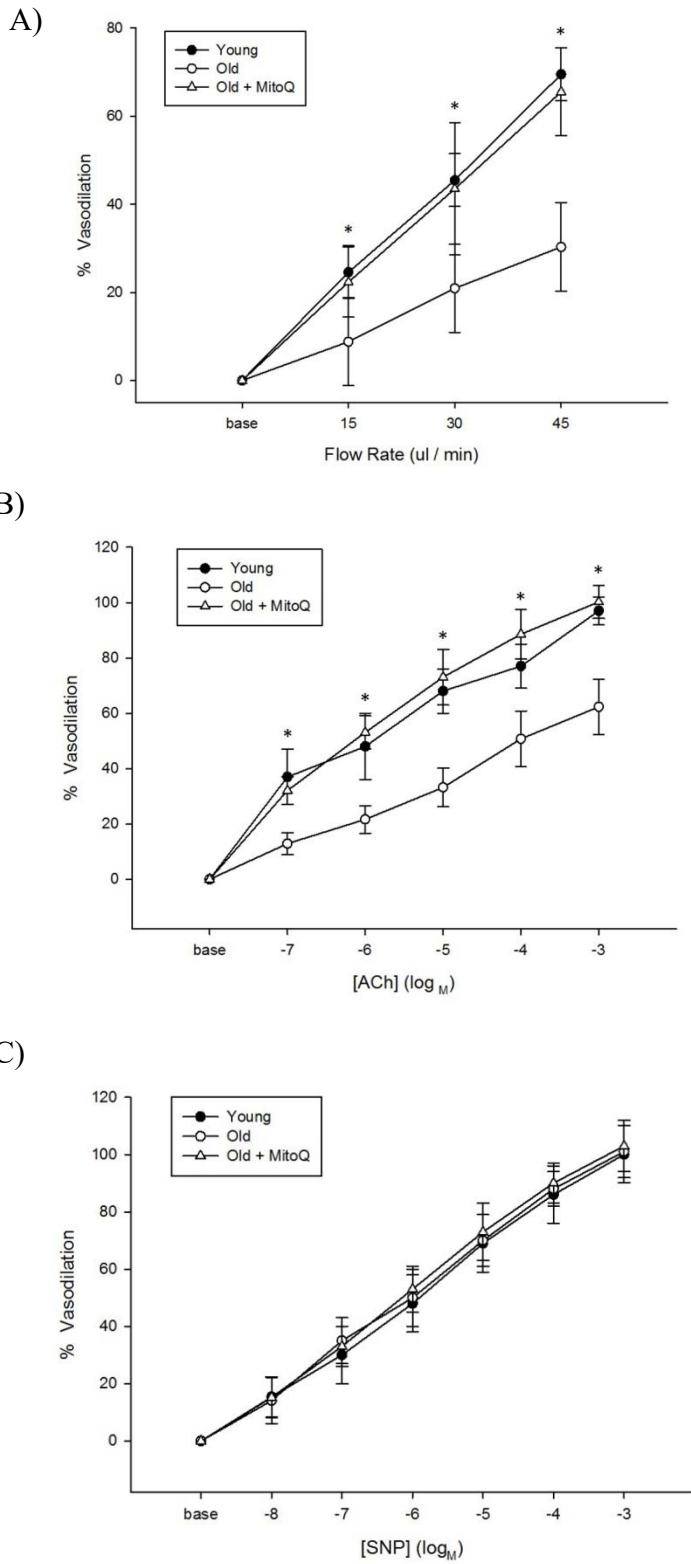


Figure 3.

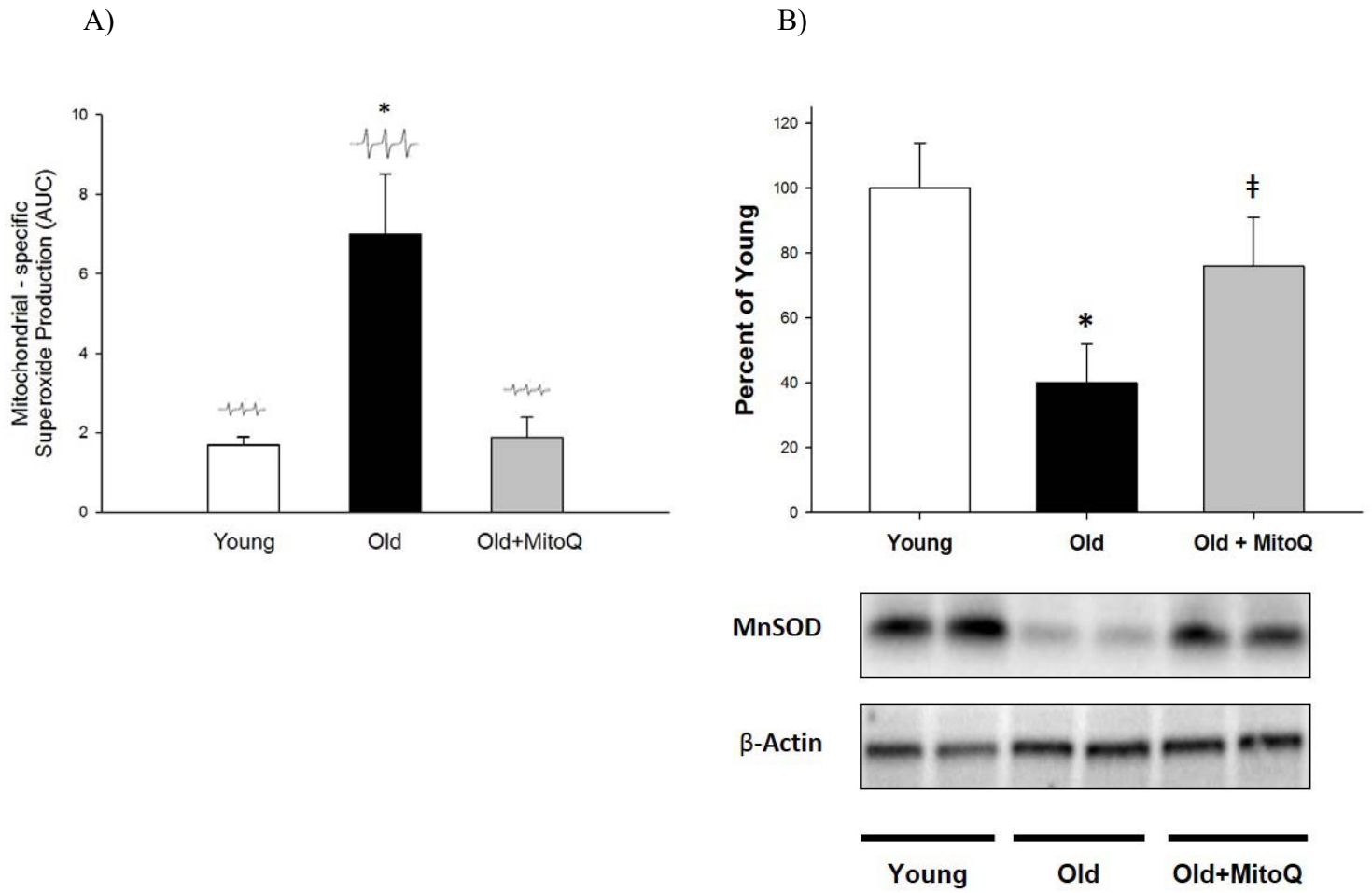
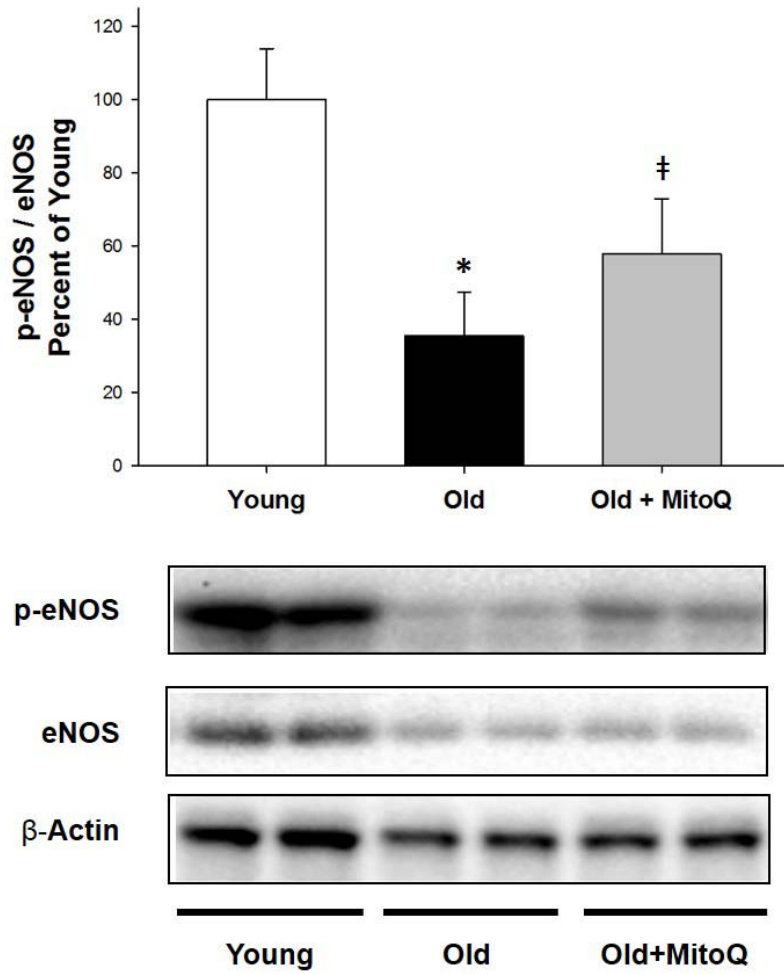


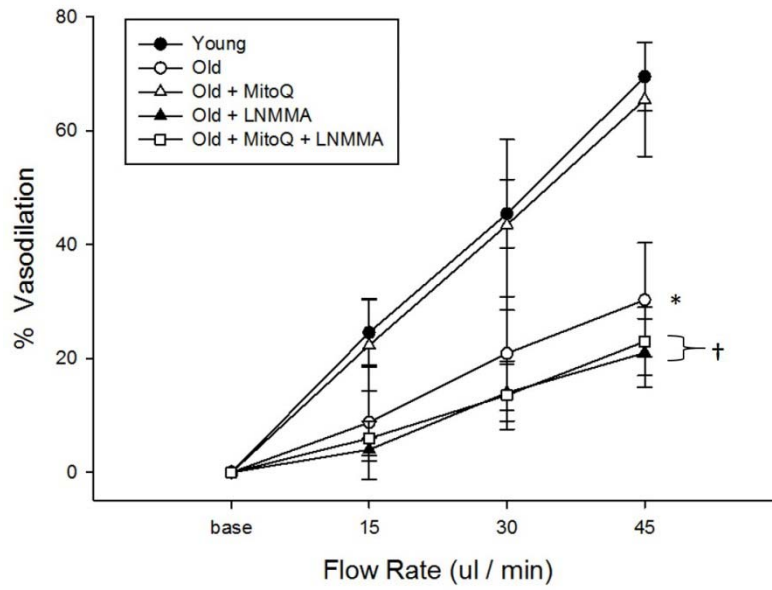
Figure 4.



1 Figure 5.

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