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Original Article

Reduced Mitochondrial Content, Elevated Reactive Oxygen Species, and Modulation by Denervation in Skeletal Muscle of Prefrail or Frail Elderly Women

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Abstract

Denervation and mitochondrial impairment are implicated in age-related skeletal muscle atrophy and may play a role in physical frailty. We recently showed that denervation modulates muscle mitochondrial function in octogenarian men, but this has not been examined in elderly women. On this basis, we tested the hypothesis that denervation plays a modulating role in mitochondrial impairment in skeletal muscle from prefrail or frail elderly (FE) women. Mitochondrial respiratory capacity and reactive oxygen species emission were examined in permeabilized myofibers obtained from vastus lateralis muscle biopsies from FE and young inactive women. Muscle respiratory capacity was reduced in proportion to a reduction in a mitochondrial marker protein in FE, and mitochondrial reactive oxygen species emission was elevated in FE versus young inactive group. Consistent with a significant accumulation of neural cell adhesion molecule-positive muscle fibers in FE (indicative of denervation), a 50% reduction in reactive oxygen species production after pharmacologically inhibiting the denervation-mediated reactive oxygen species response in FE women suggests a significant modulation of mitochondrial function by denervation. In conclusion, our data support the hypothesis that denervation plays a modulating role in skeletal muscle mitochondrial function in FE women, suggesting therapeutic strategies in advanced age should focus on the causes and treatment of denervation.

Keywords: Mitochondrial function, Myofiber size, Sarcopenia

One of the most prominent physiological changes occurring with aging is the progressive decline of physical capacity (1), which is frequently a precursor to a progressive deterioration of physical function that when severe can lead to physical frailty (2). Frailty can be described as a predisability condition that is associated with low ability to respond to physical as well as psychosocial stressors, which can eventually lead to the development of disabilities and loss of autonomy (3). Women have a higher rate of physical frailty (4), which is partly due to their higher life expectancy (5). Among the most important contributors to physical frailty are alterations in skeletal muscle that precipitate progressive weakness with advancing age (2). Although mitochondrial changes are frequently implicated in muscle alterations with aging (6), and some studies have reported greater mitochondrial impairment in elderly persons with low physical

function (7,8), little is known about mitochondrial function changes specifically in prefrail or frail elderly (FE) women.

Skeletal muscle denervation (9) and mitochondrial impairment (10) have long been considered important factors involved in age-related skeletal muscle atrophy. However, the relationship between these factors, and specifically whether there may be circumstances when mitochondrial impairment occurs secondary to muscle denervation in aging muscle, has only recently been considered in aging men (11). The rationale for considering a central role for denervation in causing mitochondrial impairment in aging muscle is based on two parallel bodies of knowledge. First, in very advanced age, there is a large accumulation of small angular fibers (11), the majority of which express a marker of denervation in a rodent model of aging (12), and wherein denervated fibers exhibit upregulation of ubiquitin ligases associated with muscle atrophy (12). Second, surgical denervation of muscle leads to elevated mitochondrial reactive oxygen species (ROS) production (13), sensitization to mitochondrial permeability transition (14), and reduced respiratory capacity (15), and these are changes commonly seen in aging skeletal muscle (16). Consistent with this rationale, we have recently shown very similar alterations in these different indices of mitochondrial function in a mouse model of sporadic denervation independent of aging (11), and that in octogenarian men muscle mitochondrial function alterations are at least partially secondary to denervation (11). Generally speaking, there is far less known about mitochondrial function changes in skeletal muscle of women with aging. Further to this point, how skeletal muscle mitochondrial function is altered and whether mitochondrial function is also modulated by denervation in FE women are unknown, but it is important to establish in the context of identifying appropriate treatment strategies as there is evidence for sex-specific differences in skeletal muscle mitochondria (17). To address this gap, we obtained biopsies from the vastus lateralis muscle of healthy young physically inactive women and FE women and measured mitochondrial respiratory capacity and ROS production in saponin-permeabilized myofibers. We hypothesized that there would be alterations in mitochondrial function in muscle of FE women and that denervation would play a modulating role in driving the changes in mitochondrial function. To our knowledge, this is the first study assessing skeletal muscle mitochondrial function in FE women.

Methods

Study Participants

A total of 22 prefrail and frail elderly women (FE; 78 ± 5 years) were recruited from the local Montreal area through local press (*Senior Times*) and by referral from the geriatric clinics of the McGill University Health Centre. The frailty syndrome was defined based on Fried criteria (18). A participant was defined frail if she presented three or more of the following criteria: (i) unintentional weight loss or low muscle mass, (ii) physical inactivity, (iii) weakness, (iv) slow walking speed, and (v) self-reported exhaustion (Supplementary Table 1). A prefrail status was defined as having one or two of the earlier-mentioned criteria. Twelve young physically inactive (YI) female controls (23 ± 3 years) were recruited from the McGill University community. They were screened for the eligibility for physical inactivity with the Godin-Shephard Leisure-Time Physical Activity Questionnaire (19) and 1-week measurement of physical activity (PA) level with Actigraph accelerometer. Participants were excluded if they reported any heart, lung, and/or central nervous system diseases; had an eating disorder; were diagnosed with dementia, depression, or diabetes; had abnormal complete blood count test; took blood thinning or anticoagulant medications; or had walking aids. Additional exclusion criteria for the young participants was being physically active (≥150 minutes/week of structured activity) or were less than 20 or more than 30 years of age. There was no attempt to restrict the study measures to a particular phase of the menstrual cycle in YI. This study was approved by the research ethics board of McGill University Health Centre (Montreal, QC, Canada; BMC-06-015; 13-211-BMB) and all participants provided written informed consent.

Skeletal Muscle Biopsy

Percutaneous skeletal muscle biopsy of the vastus lateralis muscle was performed after an overnight fast using the modified Bergstrøm needle technique with suction (20). Approximately 20–30 mg sample was used for histology and mounted with triganth gum, frozen in isopentane precooled in liquid nitrogen and stored at –80°C for muscle histology (see later). Thereafter, 60–70 mg of sample was used to assess mitochondrial function on the day of collection (see later).

Owing to limited muscle tissue from a few participants, some measurements were only performed on a subset of muscle biopsy specimens. The sample size used for each measurement is indicated in the Results section of each technique used in this study.

Muscle Immunofluorescent Labeling

Myosin heavy chains

Skeletal muscle tissue blocks were cut to 10-µm tissue sections on a cryostat (HM505E; Microm, Walldorf, Germany) at –22°C and mounted on glass slides (double frosted microscope slides; Fisher Scientific), air dried for 2 hours and stored at –80°C. Muscle cross-sections were probed using primary antibodies against the basal lamina and myosin heavy chain (MHC) isoform proteins. Slides were first removed from –80°C and allowed to air dry for 1 hour at room temperature. Sections were hydrated with 1× phosphate-buffered saline (PBS) and blocked with 10% normal goat serum in 1× PBS for 30 minutes. Sections were next incubated for 1 hour at room temperature with the following primary antibodies: polyclonal rabbit anti-laminin IgG (L9393, 1:700; Sigma-Aldrich), monoclonal mouse anti-MHC I IgG2b (BA-F8, 1:25), monoclonal mouse anti-MHC IIa IgG1 (Sc71, 1:200), and monoclonal mouse anti-MHC IIx IgM (6H1, 1:25). All MHC primary antibodies were purchased from Developmental Studies Hybridoma Bank (University of Iowa, IA). After incubation, tissue sections underwent a series of 1× PBS washes and incubation with fluorescently labeled secondary antibodies for 1 hour at room temperature: Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:500), Alexa Fluor 350 goat anti-mouse IgG2b (A21140, 1:500), Alexa Fluor 594 goat anti-mouse IgG (A21125, 1:100), and Alexa Fluor 488 goat anti-mouse IgM (A21042, 1:500). All secondary antibodies were obtained from Thermo Fisher Scientific. After incubation, muscle cross-sections were washed 3 × 5 minutes in 1× PBS and mounted with coverslips using Prolong Gold mounting medium (Invitrogen, Thermo Fisher Scientific). Images of the stained tissue sections were acquired with a Zeiss fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany) and analyzed with ImageJ (National Institute of Health, Bethesda, MD). A minimum of 100 fibers were sampled per muscle section and analyzed for their shape and cross-sectional area. Atrophied fibers were determined based on the first percentile in YI controls. The first percentile was determined following the calculation of muscle fiber size distribution of all fibers for all YI subjects, where the first percentile size corresponded to fibers with a size of less than or equal to 1,441 µm^2.

Neural cell adhesion molecule

In serial muscle cross-sections, we labeled with a primary antibody against neural cell adhesion molecule (NCAM; AB5032; 1:100; Millipore Sigma) to provide an indication of the degree of denervation in YI versus FE muscle. Tissue slides were fixed in 4% paraformaldehyde, washed with 1× PBS and permeabilized with 0.1% Triton-X. After blocking, slides were incubated with anti-NCAM antibody and anti-MHC I antibody overnight at 4°C. The next day, tissue sections were washed with 1× PBS and incubated with fluorescence-labeled secondary antibodies for 1 hour at room temperature. For NCAM Alexa Fluor 594 (1:200) and MHC I Alexa Fluor 350 (1:500) were used. A serial tissue section was stained for laminin as described earlier.

Mitochondrial Function Measurement

Mitochondrial function was measured as described previously (11). Briefly, muscle tissue was immediately placed in ice-cold buffer A (Supplementary Table 2) for manual dissection under a stereomicroscope. Muscle tissue was manually dissected and separated into small bundles (2–6 mg) of muscle fibers. Bundles of muscle fibers were subsequently placed in ice-cold buffer A with 0.05 mg/mL of saponin to allow chemical permeabilization for 30 minutes at low rocking speed.

High-Resolution Respirometry

Following 30-minute saponin permeabilization, myofibers were washed 3 × 10 minutes in buffer B (Supplementary Table 2). Oxygen consumption was subsequently measured using a high-resolution respirometer (Oxygraph-2k; Oroboros, Innsbruck, Austria). Polarographic oxygen sensors were calibrated by a two-point calibration for air saturation and zero oxygen concentration. Thereafter, 3–5 mg of wet weight permeabilized myofibers were added to 2 mL buffer B in the respirometer and equilibrated at 37°C with constant stirring. Respiration was conducted under hyperoxygenated conditions to limit oxygen diffusion limitation. Oxygen flux was assessed by the titration of substrates, where each addition was followed by a short period of stabilization. To obtain state 2, respiration glutamate (10 mM) and malate (5 mM) were simultaneously added to the chamber. Next, with the titration of adenosine diphosphate (ADP; 2 mM), adenosine triphosphate synthesis was stimulated and maximal state 3 respiration driven by complex I substrates was achieved. To assess state 3 respiration with both complex I and complex II substrates, the complex II substrate succinate (10 mM) was added to the chamber. To check the integrity of the outer mitochondrial membrane, cytochrome *c* (5 mM) was added to the substrate mix, wherein an increase in respiration following addition of exogenous cytochrome *c* signifies damage to the outer mitochondrial membrane. Next, we added antimycin A (10 µM) to inhibit complex III, and with the titration of ascorbate (10 mM) and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD; 1 mM) we assessed complex IV respiratory capacity independent of the upstream complexes. Values obtained by TMPD-stimulated respiration were corrected for autooxidation by TMPD, as done previously (11). After these measurements, the degree of mitochondrial respiration coupling was assessed by calculating the acceptor control ratio (state III respiration with GM substrates/state II respiration with GM substrates). After respiration was completed, muscle bundles were frozen in liquid nitrogen and stored at –80°C for protein measurement.

Measurement of Mitochondrial ROS Emission

To measure ROS emission, myofibers were washed 3 × 10 minutes in buffer Z (Supplementary Table 2). ROS production was detected by recording the rate of generation of the fluorescent compound resorufin from Amplex Red. Resorufin is formed by the reaction of Amplex Red and hydrogen peroxide as well as fatty acid hydroperoxides (FA-OOH) released from the mitochondria and catalyzed by horseradish peroxidase. A fluorescence spectrophotometer (Hitachi F-2500, Tokyo, Japan) was used to measure the fluorescence signal at an excitation/emission wavelength of 563/587 nm. The fluorescent signal was measured with the FL Solutions Software (Hitachi High Technologies Corporation, Tokyo, Japan). Approximately 3–6 mg of muscle fiber bundles were added to 600 µL of solution Z, with 5.5 µM Amplex red and 1 U/mL horseradish peroxidase in a glass cuvette at 37°C and with a magnetic stirrer. After a short period of baseline autofluorescence, muscle fibers were added into the cuvette and the following substrates were titrated: glutamate (10 mM) + malate (2 mM), succinate (10 mM), ADP (10 µM), ADP (100 µM), and antimycin A (10 µM).

Measurement of Denervation-Induced Mitochondrial ROS Emission

It has been established previously that there is an increase calcium-dependent phospholipase A_2 ($cPLA_2$) activity in denervated muscle that causes the release of ROS from mitochondria in the form of FA-OOH and that these FA-OOH are detected with Amplex Red (21). As the denervation-induced ROS response detected by Amplex Red is specific to $cPLA_2$ activity rather than ROS emission in general, it can be used as a means of identifying the influence of denervation on mitochondrial function, as shown in mouse models of sporadic denervation such as the SOD1 knockout and SODG93A amyotrophic lateral sclerosis models (21), and the neurotrypsin overexpressing mouse model (11). Thus, to determine whether denervation is modulating mitochondrial function we inhibited the activity of $cPLA_2$ with the application of arachidonyl trifluoromethyl ketone ($AACOCF_3$), as previously described by Bhattacharya and colleagues (21) and used in our recent study of elderly men (11). To confirm the specificity of the ROS response to the $cPLA_2$ isoform, and thus, to the influence of denervation, we also applied bromoenol lactone, a specific inhibitor of the calcium-independent PLA_2 isoform ($iPLA_2$) that is associated with muscle ROS production in the context of disuse muscle atrophy (22), to a subset of samples. A reduction in Amplex Red signal (resorufin) with $cPLA_2$ inhibition but not $iPLA_2$ inhibition is interpreted as evidence for modulation of mitochondrial function by denervation (21). Saponin-permeabilized muscle fibers were washed 3 × 10 minutes in buffer Z with $AACOCF_3$ (20 µM) and buffer Z with ethanol (vehicle control). The Amplex Red signal was measured as mentioned previously in the presence or absence (ethanol vehicle only) of $AACOCF_3$, to assess the contribution of FA-OOH to the endogenous ROS production.

After mitochondrial function measurements were completed, muscle bundles were frozen in liquid nitrogen and stored at –80°C for protein measurement. All mitochondrial function experiments were analyzed using an in-house analysis program created using Igor Pro Software (Wavemetrics; https://www.wavemetrics.com).

Mitochondrial Content Estimation

Skeletal muscle fibers that were used in the mitochondrial function assays were subsequently probed for protein levels of voltage-dependent anion channel (VDAC) by western blotting, where the content of the VDAC (an outer membrane protein) was used as a general indicator of mitochondrial content. Between 6 and 35 mg of muscle was homogenized 2 × 45 seconds with a robot homogenizer (Minibeadbeater, Biospec Products) with a 10× volume of radioimmunoprecipitation

assay buffer extraction buffer (Supplementary Table 2). For homogenization, 1.4 mm ceramic beads were used. After 2-hour incubation at 4°C, the samples were centrifuged at 12,000*g* for 20 minutes at 4°C. The supernatant was removed and used to measure protein content with the Bradford assay. Immunoblotting was performed with 15 µg tissue protein diluted in 4× Laemmli buffer and extraction buffer and boiled at 95°C for 5 minutes. Twenty-four microliter sample was then loaded onto a 12% acrylamide gel, electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred at 4°C to polyvinylidene fluoride membranes (Amersham Hybond ECL; GE Healthcare Life Sciences). The membranes were blocked in 5% semi-skimmed milk for 1 hour at room temperature and incubated overnight at 4°C with a primary mouse monoclonal anti-VDAC antibody (ab14734, 1:1,000; Abcam) diluted in 5% bovine serum albumin. Because β-actin, glyceraldehyde 3-phosphate dehydrogenase, and α-tubulin tend to decrease with age, total protein loading was used to normalize protein levels (23). Membranes were incubated with rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody diluted in 5% milk (ab6728, 1:2,000; Abcam) for 1 hour at room temperature. Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) and imaged with a Bio-Rad imaging system (ChemiDoc MP Imaging System). Identification of VDAC was performed using Image Lab Software, where both bands at the approximate molecular mass of VDAC in a given blot were summed to obtain an index of the quantity of VDAC protein (23).

Statistical Analysis

In all cases except ROS emission for the denervation-induced ROS estimation, the comparison between YI and FE groups was performed with an unpaired two-tailed Student's *t* test. The presence of outliers was tested with the ROUT method in GraphPad Prism 7. Results are expressed as mean ± standard deviation (*SD*). Differences between groups were considered statistically significant at *p* value less than .05. Differences in the Amplex Red signal during the denervation-induced ROS estimation between ethanol controls and either $AACOCF_3$ or bromoenol lactone treatment were analyzed by paired *t* test. Data were statistically analyzed using GraphPad Prism 7 for Microsoft Windows.

Results

Study Participants

Based on the Fried criteria of frailty (18), 8 participants were characterized as prefrail and 14 as frail. The main physical characteristics of the participants are summarized in Table 1. Two participants in the prefrail group had low PA as the only parameter meeting the prefrail criteria (data not shown). The measurement of PA is not well standardized (3), and it has been suggested, therefore, that low PA alone is not a good indicator of the prefrail status. However, because their age and other data (described later) fit with other subjects in the prefrail or frail group, the inclusion of these two participants does not affect the conclusions about the FE group. Both age groups were similar in weight, height, and body mass index ($p > .05$). Within the FE group, there were no differences between prefrail and frail subjects in terms of myofiber size, mitochondrial respiration capacity, and ROS production, hence, prefrail and frail subjects were combined into FE group.

Table 1. Descriptive Characteristics of YI and FE Participants

Characteristics	YI (n = 12)	FE (n = 22)
Age (y)	24.0 ± 3.5	78.0 ± 5.9***
Height (cm)	162.5 ± 0.1	156.9 ± 7.7*
Weight (kg)	61.8 ± 11.9	64.1 ± 10.0
BMI (kg/m^2)	23.5 ± 4.6	26.2 ± 4.7

Note: Values presented as mean ± *SD*. BMI = body mass index; FE = prefrail or frail elderly; YI = young inactive.
*$p < .05$, ***$p < .001$.

Muscle Fiber Size and Size Distribution

Figure 1 shows representative micrographs of muscle cross-sections from YI and FE (A), revealing a reduced mean fiber size (B), and a marked abundance of very small muscle fibers (≤1,441 µm^2) in limb muscle of the FE group (C).

Mitochondrial Content and Respiration

VDAC was used as a marker for mitochondrial content. Representative western blots are depicted in Figure 2A. The abundance of the VDAC protein was significantly lower in the FE group (n = 21) compared to the YI group (n = 12) (Figure 2B; p = .004). Whereas state 3 respiratory capacity of complex I (GM + ADP) did not reach statistical difference between the groups (p = .07), state 3 respiration driven by complex I and II substrates (GM + ADP + succinate) was significantly lower in the FE group (Figure 3A; p = .019). In addition, we found that the respiratory capacity of complex IV was significantly lower in FE group versus YI group (Figure 3A; p = .003), as assessed using the artificial electron donor TMPD following inhibition of complex III using antimycin A.

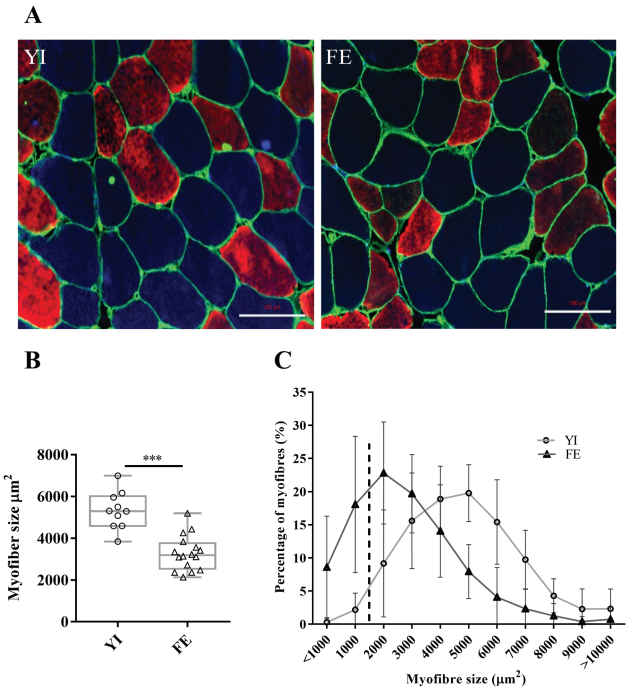

Figure 1. Skeletal muscle fiber morphology in vastus lateralis muscle of young inactive (YI) and prefrail or frail elderly (FE). (**A**) Skeletal muscle cross-sections immunolabeled for laminin (cell membrane), type I, type IIa, and type IIx myosin heavy chain isoforms. (**B**) Mean fiber cross-sectional area (YI, n = 10; FE, n = 16). (**C**) Fiber size distribution. The dashed vertical line represents fibers that were atrophied (YI 1.3%; FE 15.2%) determined based on the first percentile in YI controls. Graphs show mean ± *SD*. Scale bars = 100 µm. ***$p < .001$. Full color version is available within the online issue.

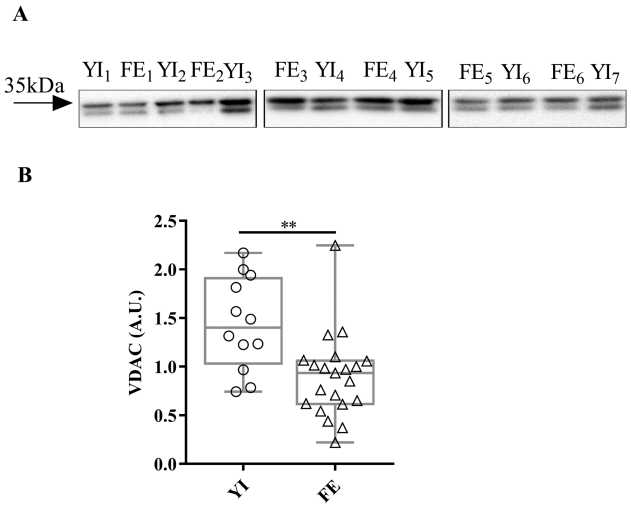

Figure 2. Changes in mitochondrial content in human muscle. (**A**) Representative sections from three separate western blots showing voltage-dependent anion channel (VDAC) protein. Each section from a separate western blot is specifically outlined. (**B**) Abundance of VDAC protein in vastus lateralis muscle of young inactive (YI) and prefrail or frail elderly (FE) participants with corresponding western blot (YI, n = 12; FE, n = 21). Both bands at the approximate molecular weight of VDAC were summed to represent VDAC content. Graphs show mean ± *SD*. $^{**}p < .01$.

Next, we evaluated mitochondrial respiratory capacity after normalization to mitochondrial content (VDAC protein) and found this abolished the differences in respiratory capacity between groups (Figure 3B), suggesting that the reduced muscle respiratory capacity in FE was a function of lower mitochondrial content. We then assessed the oxidative phosphorylation coupling, by the respiratory acceptor control ratio, and found a significantly lower acceptor control ratio in the older group (Figure 3C; p = .028). Note that based on a more than 10% increase in respiration with the addition of exogenous cytochrome *c* following the succinate step, the integrity of the outer mitochondrial membrane was judged to be interrupted in five cases (FE, n = 3; YI, n = 2). These samples were excluded from the mitochondrial respiration capacity data set reported earlier.

Mitochondrial ROS Production

ROS emission under state 2 and state 3 respiration when normalized to wet weight did not differ between groups (Figure 4A; FE, n = 20; YI, n = 12; G + M; + succinate; $p > .05$). After mitochondrial ROS emission rates were expressed relative to mitochondrial content (VDAC protein), the FE group demonstrated a significantly greater ROS emission under state 1 respiration as well as under state 2 and state 3 respiration compared to the YI group (Figure 4B; FE, n = 20; YI, n = 12; fibers, p = .02; G + M, p = .001; +succinate, p = .002; ADP 0,1 M, p = .009; ADP 1 M, p = .008), revealing a greater intrinsic mitochondrial ROS emission in FE.

Impact of Muscle Denervation on Mitochondrial Function

To establish the extent of denervation in our sample, we labeled muscle cross-sections with an antibody against the denervation-responsive cytokine, NCAM. As shown in Figure 5A and B, there was a pronounced increase in the abundance of muscle fibers that were positive for NCAM labeling in the FE group (p = .039). Following up on this, to determine if muscle denervation was influencing mitochondrial function in our measures we used a pharmacological approach

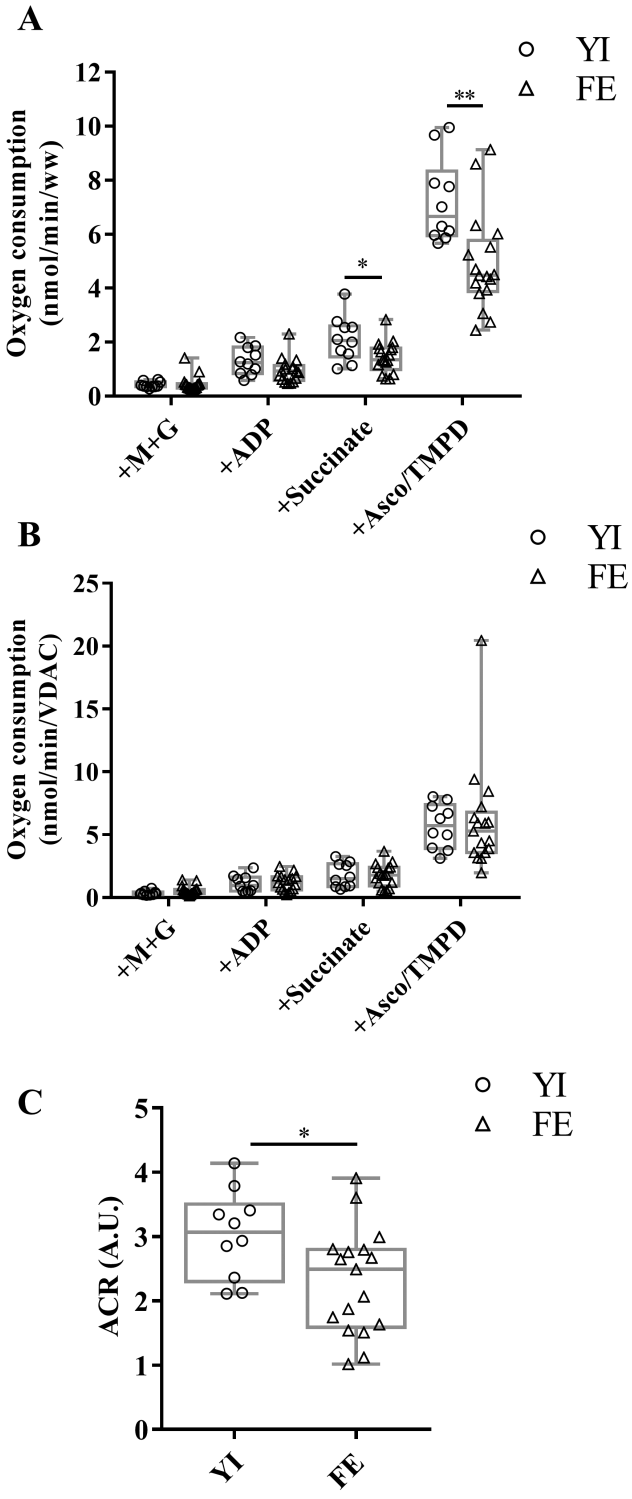

Figure 3. Mitochondrial respiratory capacity in permeabilized muscle fiber bundles. (**A**) Oxygen consumption measured in young inactive (YI; n = 10) and prefrail or frail elderly (FE; n = 17) group normalized to fiber wet weight. (**B**) Oxygen consumption normalized to the abundance of voltage-dependent anion channel (VDAC) protein. (**C**) Differences in mitochondrial oxidative phosphorylation coupling between YI and FE group. Graphs show mean ± *SD*. $^{*}p < 0.05$; $^{**}p < 0.01$. ACR = acceptor control ratio.

with the application of the $cPLA_2$ inhibitor $AACOCF_3$ to inhibit the denervation-induced mitochondrial ROS response (21), as we have done previously in octogenarian men and a mouse model of sporadic

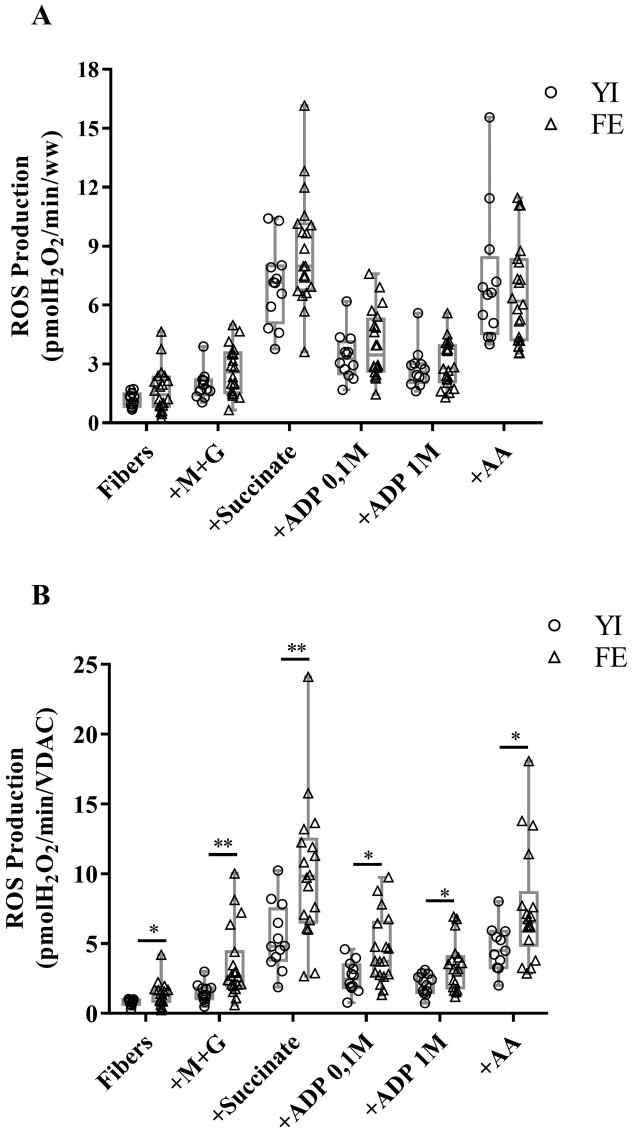

Figure 4. Mitochondrial reactive oxygen species (ROS) production in permeabilized muscle fiber bundles. (**A**) Differences in ROS signal between young inactive (YI; $n = 12$) and prefrail or frail elderly (FE; $n = 20$) group when normalized to wet weight. (**B**) Differences in ROS signal between groups (YI, $n = 12$; FE, $n = 20$) when normalized to the abundance of voltage-dependent anion channel (VDAC) protein. Graphs show mean ± *SD*. $^{*}p < .05$; $^{**}p < .01$.

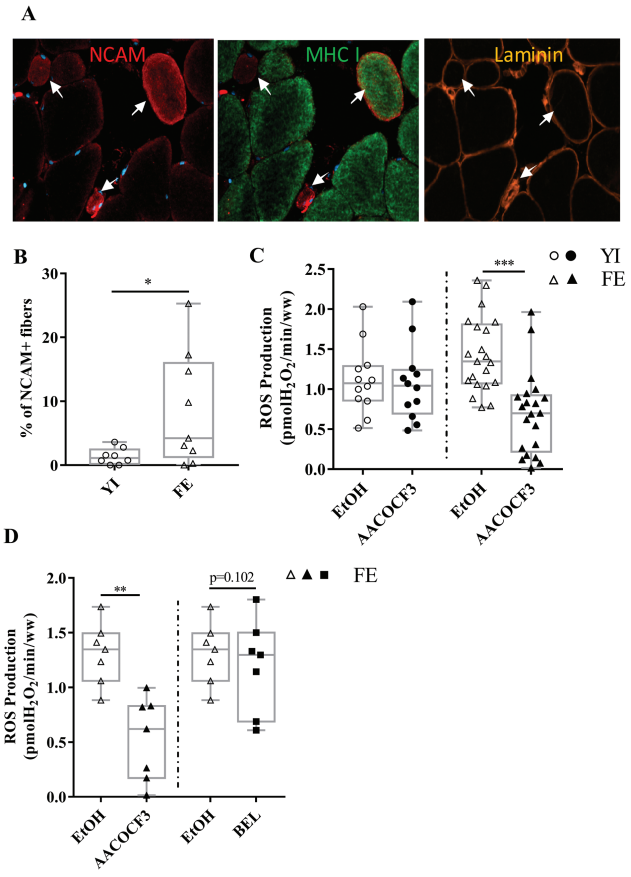

Figure 5. Evidence of denervation and its modulating role on mitochondrial function. (**A**) Representative images of a section labeled with neural cell adhesion molecule (NCAM), myosin heavy chain (MHC) I, and laminin from a prefrail or frail elderly (FE) subject. (**B**) Plot of the abundance of NCAM-positive muscle fibers in young inactive (YI; $n = 8$) and FE ($n = 9$) groups. (**C**) Changes in reactive oxygen species (ROS) signal compared between vehicle control (ethanol) and the addition of the cytoplasmic phospholipase A_2 (cPLA$_2$) inhibitor, application of arachidonyl trifluoromethyl ketone (AACOCF$_3$) in the older group (YI, $n = 12$; FE, $n = 21$). (**D**) Changes in ROS signal among FE ($n = 7$) with the addition of the cPLA$_2$ inhibitor AACOCF3 compared to the addition of the calcium-independent phospholipase A2 (iPLA$_2$) inhibitor bromoenol lactone (BEL). Graphs show mean ± *SD*. $^{*}p < .05$. Full color version is available within the online issue.

denervation (11). cPLA$_2$ inhibition significantly reduced ROS production compared to ethanol vehicle in FE ($n = 21$; Figure 5C; $p < .001$) but had no effect on ROS production in the YI group ($n = 12$; Figure 5C; $p = .403$). In contrast to what was observed with cPLA$_2$ inhibition in FE, use of the iPLA2 inhibitor bromoenol lactone had no effect on ROS production in a subset of FE group (Figure 5D), showing that a reduction in ROS emission in FE was specific to the contribution of ROS from the denervation-induced cPLA$_2$ isoform (20).

Discussion

Skeletal muscle is abundant in mitochondria that help to provide the energy required for normal muscle function. Furthermore, mitochondria are also involved in homeostasis (eg, pH, Ca^{2+}), apoptotic signaling and are an important source of ROS generation in the cell (10). Alterations in these processes occur in association with age-related skeletal muscle atrophy (16). Muscle mass loss, exacerbated muscle fatigue, and reduced muscle strength are key components of the frailty syndrome, hence mitochondria have also been implicated in the frailty syndrome (8,24,25). Nevertheless, although women are at a greater risk of becoming frail, there is limited information about mitochondrial alterations in skeletal muscle of elderly women. Furthermore, it has been recently shown that skeletal muscle mitochondrial function alterations in octogenarian men are at least partly secondary to myofiber denervation (11), and this has not yet been addressed in FE women. As such, the purpose of this study was to assess mitochondrial function changes in skeletal muscle, and the potential modulating impact of denervation, in FE women.

Mitochondrial Function in Aging Muscle

Several studies have shown declines in the activity of various mitochondrial enzymes in older women (26–28), but only one has assessed this issue in frail elderly women, finding a lower phosphocreatine recovery time by magnetic resonance spectroscopy and lower mitochondrial respiratory complex activities (29). Interestingly, another study found

that elderly women with lower mitochondrial DNA (mtDNA) copy number assessed in blood samples were more likely to be frail (25). This group has also identified a specific mtDNA variant, which is associated with the control region of mtDNA replication that is suggested to alter mtDNA copy number in the frail population (25,30). Although the cause and effect relationship could not be assessed, these findings also support the potential role of mitochondria in the frailty syndrome. Another study in frail women found reduced levels of nuclear respiratory factor 1 transcript in skeletal muscle tissue when compared with healthy active elderly women (24), which may indicate that frail women have altered mitochondrial biogenesis secondary to reduced PA. A study that assessed various aspects of mitochondria in skeletal muscle of three generations of women found that elderly women had lower citrate synthase activity and mtDNA copy number, whereas indices of mitochondrial biogenesis were not affected (27). The same study found that mtDNA mutations may not be the main contributor to age-related mitochondrial dysfunction, based on the deletions being present in the noncoding regions of the mtDNA (27). Elderly women also appear to have reduced mitochondrial size, as assessed by electron microscopy, suggesting a degree of mitochondrial fragmentation (31). Whereas one study in very old (mean age of 83 years) physically inactive women observed a tendency to reduced transcript levels of mitofusin 2 (mfn2) and dynamin-related protein 1 (Drp1), suggesting impaired mitochondrial fission and fusion signaling (24), another study examining a cohort consisting of 45% elderly women reported no changes in protein abundance of mitofusion 1, Drp1 or fission 1 protein (Fis1), but a significant decline in optic atrophy gene 1 (OPA1) (8). In the only previous study to examine mitochondrial function directly, Joseph and colleagues (8) reported lower state 3 respiratory capacity in saponin-permeabilized myofibers obtained from a mixed cohort of low-functioning elderly men and women (45% women). Our results are generally consistent with this finding, wherein we observed a reduced mitochondrial respiratory capacity in FE women and, based on a lower VDAC protein level, this appears to be due to reduced mitochondrial content rather than intrinsic mitochondrial respiratory capacity impairment. We acknowledge, however, that a single mitochondrial protein may not represent the dynamics of the hundreds of proteins that comprise a mitochondrion and that the gold standard measure here is mitochondrial volume density. In this respect, in the only study to have specifically made this measure in older women only a trend to a reduction in intermyofibrillar mitochondrial volume density was seen in moderately physically active women aged less than 70 compared to activity-matched young women (31). Because our FE subjects were approximately a decade older and quite sedentary by comparison to the older women in this previous study (31), a reduced muscle mitochondrial content in FE women as suggested by our VDAC measures is not unexpected. We also note that in other experiments we did not observe a difference in the relative abundance of type I versus type II fibers between groups (data not shown), so fiber type does not appear to relate to the differences in mitochondrial abundance in the YI versus FE groups. The FE group also displayed lower acceptor control ratio, suggesting a reduced mitochondrial oxidative phosphorylation coupling efficiency, which may be an adaptive response to limit age-related increased generation of mitochondrial ROS (32). Thus, our findings add to the previous studies noted herein and support the notion that mitochondrial alterations are an important component of frailty in elderly women, and muscle alterations in aging women in general.

Mitochondrial ROS Production

Although numerous studies have shown that mitochondrial ROS increases in various conditions involving muscle atrophy (13,33), only some studies have reported that mitochondrial ROS is elevated in aging atrophied muscle (34,35), whereas others have shown no increase despite muscle atrophy (36,37) or a highly variable response independent of atrophy (38). On the other hand, we have previously shown that oxidative damage is highly heterogeneous between individual muscle fibers in aged muscle (39), and thus increases in mitochondrial ROS in a subset of muscle fibers (eg, such as those that are denervated (13)) could be obscured by the ROS signal in the surrounding muscle fibers. Notwithstanding this point, because mitochondria are a main site of ROS production in the cell, mitochondrial impairment has been suggested to play a role in frailty. Consistent with this notion, it has been shown that skeletal muscle from elderly individuals with reduced physical function is more susceptible to oxidative damage (8). Various studies have also presented an association between oxidative stress and frailty by measuring circulating biomarkers in plasma samples (40–42), wherein frail individuals presented increased protein and glutathione oxidation, reduced levels of total thiol, elevated levels of isoprostanes, as well as oxidative DNA damage. Another study assessed the presence of oxidative stress more directly, observing an increased production of superoxide anions in white blood cells from frail individuals (43). Interestingly, to the best of our knowledge, none of the studies in frail individuals measured ROS in skeletal muscle. Thus, our study shows for the first time that ROS emission in vastus lateralis muscle is markedly higher in the FE group after normalization to mitochondrial content, suggesting that mitochondria in limb muscle of very old prefrail or frail women have features that potentiate ROS production.

Sex-specific Changes in Mitochondrial Function With Aging

The current literature is rich with studies assessing changes in mitochondrial function with aging; however, most of these studies do not account for possible difference between sexes. Recent research suggests that there is significant between-sex dimorphism in mitochondrial physiology in healthy adults (17). Indeed, studies have found that, compared to men, women had reduced sensitivity to ADP (44), higher mitochondrial volume density (45), and increased lipid oxidation (44,45). However, there were no sex-dependent differences found in mitochondrial maximal oxidative phosphorylation of vastus lateralis (44) and gastrocnemius muscles (46), suggesting other factors may contribute to sex-dependent differences in mitochondrial function, such as posttranscriptional modifications or allosteric enzyme regulation. It should be noted that participants in the studies noted earlier were young or middle-aged individuals, and thus mitochondrial functional differences with aging may not yet be apparent. Hence, our study could provide a foundation for a better understanding of sex-specific age-related changes in mitochondrial function by making some comparisons of the FE women studied here to our previous study of octogenarian men (11). In this respect, it is noteworthy that whereas our current study finds proportional changes in muscle respiratory capacity and VDAC protein in FE versus YI women, our prior study (11) found no significant reduction in muscle respiratory capacity or VDAC protein with aging in octogenarian men. This suggests women but not men exhibit a decline in mitochondrial content in advanced age. In contrast, our prior study (11) found no increase in mitochondria-specific ROS emission in octogenarian men, whereas our current results show an increase in mitochondria-specific ROS emission in FE women. This latter result suggests women but not men exhibit a mitochondria-specific exacerbation of ROS generation in advanced age. Interestingly, these differences occurred despite the evidence that denervation was modulating mitochondrial function in both octogenarian men (11) and the FE women studied here (see

Muscle Denervation section). In addition, we cannot rule out the possibility that the frailty status of the current cohort of FE women was more severe than that of the octogenarian men reported previously. Thus, the biological basis for these sex-dependent differences cannot be revealed by our analysis and additional study of sex-specific variation in skeletal muscle mitochondrial function and its impact on skeletal muscle mass and function is clearly warranted.

Muscle Denervation

Frail individuals appear to have exacerbated alterations in the central nervous system indicated by increased prevalence of dementia (47), a neurological disorder that affects neuronal connectivity of the brain (reviewed in ref. (48). Hence, it is possible that they may also exhibit exacerbated motor neuron degeneration, which is known to contribute to age-related muscle atrophy (11,12). Consistent with this notion, we observed a marked increase in the abundance of muscle fibers positive for the denervation-responsive cytokine, NCAM, in FE muscle.

Muscle fibers undergo repeated cycles of denervation and reinnervation for much of adult life; however, in very old age (>75 years of age in humans) persistently denervated fibers start to accumulate and often occur as grouped fiber atrophy, implicating loss of an entire motor unit or a large axon innervating multiple fibers (9). Studies in animal models suggest that failed reinnervation of muscle fibers is responsible for the accumulation of persistently denervated fibers in advanced age (12,49) and that this is a key contributor to an acceleration of atrophy in very old age (50). Interestingly, Bhattacharya and colleagues (21) have previously shown that denervation increases mitochondrial ROS emission in the form of FA-OOH cleaved from the outer mitochondrial membrane by the enzyme $cPLA_2$ and that the denervation-induced ROS signal can be ablated by inhibiting $cPLA_2$. We have also shown in both a mouse model of sporadic denervation and octogenarian men, whose muscle exhibited morphological and transcriptional changes indicative of denervation, that $cPLA_2$ inhibition reduces ROS emission (11), showing that this method can be used to uncloak the impact of denervation on mitochondrial function. As mentioned in the Sex-specific Changes in Mitochondrial Function With Aging section, these latter observations in octogenarian men are consistent with this study where we found a significant reduction in mitochondrial ROS emission following $cPLA_2$ inhibition in FE women but not YI women. We further showed that the reduction in ROS emission occurred only with $cPLA_2$ inhibition and not with $iPLA_2$ inhibition in fibers from FE, which based on Bhattacharya and colleagues's previous results showing that the denervation-induced ROS response is specific to $cPLA_2$ action, indicates that denervation is modulating the ROS response in FE. Finally, similar to our previous study in octogenarian men (11), the modulation of mitochondrial function by denervation in FE women was accompanied by a significant accumulation of severely atrophied muscle fibers, as is typical of persistent denervation in aging muscle (12,49). Thus, our results support our hypothesis that denervation contributes to mitochondrial impact in limb muscle of FE women. Although this could suggest that exacerbated muscle denervation may be a risk factor for frailty, it is not possible with this study design to discern causality in this relationship. Thus, a more comprehensive study in elderly women including very old nonfrail participants is needed.

Conclusions

Loss of muscle mass and function are major contributors to physical frailty, and therefore, intervening with these processes could be effective in reducing physical frailty. In this respect, our results show that denervation is an important modulator of mitochondrial function in FE women as demonstrated by the reduction in mitochondrial ROS emission after $cPLA_2$ inhibition. This is consistent with the histological presence of very small fibers typical of persistent denervation in skeletal muscle cross-sections from elderly women. In addition, it would be valuable to determine whether the reduction in mitochondrial content suggested by lower VDAC that we observed in FE group could be prevented through PA, as suggested by other studies. As such our results add to the existing data in octogenarian men and confirm that denervation is present in both elderly men and women, suggesting that when aging muscle atrophy becomes clinically relevant denervation rather than the mitochondrion may be a more effective therapeutic target.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of interests

None.

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