Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers

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Summary

Mitochondria regulate cellular bioenergetics and apoptosis and have been implicated in aging. However, it remains unclear whether age-related loss of muscle mass, known as sarcopenia, is associated with abnormal mitochondrial function. Two technically different approaches have mainly been used to measure mitochondrial function: isolated mitochondria and permeabilized myofiber bundles, but the reliability of these measures in the context of sarcopenia has not been systematically assessed before. A key difference between these approaches is that contrary to isolated mitochondria, permeabilized bundles contain the totality of fiber mitochondria where normal mitochondrial morphology and intracellular interactions are preserved. Using the gastrocnemius muscle from young adult and senescent rats, we show marked effects of aging on three primary indices of mitochondrial function: respiration, H2O2 emission, sensitivity of permeability transition pore to Ca2+ when measured in isolated mitochondria, but to a much lesser degree when measured in permeabilized bundles. Our results clearly demonstrate that mitochondrial isolation procedures typically employed to study aged muscles expose functional impairments not seen in situ. We conclude that aging is associated with more modest changes in mitochondrial function in sarcopenic muscle than suggested previously from isolated organelle studies.

Key words: isolated mitochondria; skinned fibers; aging; skeletal muscle; sarcopenia.

Introduction

Mitochondria play central roles in the regulation of cellular metabolism (Lesnefsky & Hoppel, 2006) and apoptosis (Manoli et al., 2007; Wenz et al., 2009). Likewise, several metabolic changes occur with muscle atrophy (Lecker et al., 2004), and recent evidence suggests that mitochondrial function can be a key regulator of the atrophic process (Romanello et al., 2010). For this and other reasons, age-related changes in mitochondrial function have been implicated in the decline of muscle mass and function with aging known as sarcopenia (Wanagat et al., 2001; Terman & Brunk, 2004; Hiona & Leeuwenburgh, 2008). The isolation of mitochondria from skeletal muscle is a widely employed method in studies examining mitochondrial function under various conditions, including aging (Frezza et al., 2007b; Lanza & Nair, 2009). Such studies have demonstrated significant alterations in a variety of indices of mitochondrial function in aged skeletal muscles including reduced maximal ATP-generating capacity (Drew et al., 2003), reduced maximal respiratory capacity (Chabi et al., 2008), increased reactive oxygen species (ROS) generation (Capel et al., 2004; Mansouri et al., 2006; Muller et al., 2007), and impaired function of the mitochondrial permeability transition pore (mPTP) (Seo et al., 2008). While these studies appear to reveal physiologically relevant alterations in mitochondrial function, the methods employed do not take into consideration the complex structural arrangement of mitochondria in vivo (Bakeeva et al., 1978; Ogata & Yamashiki, 1997) nor the potential for the isolation process to expose vulnerabilities in aged mitochondria that are not evident in vivo. Mitochondria are dynamic organelles that exhibit varying degrees of a mitochondrial network in skeletal muscle (Kayar et al., 1988; Ogata & Yamashiki, 1997; Shaw et al., 2008), and this network facilitates functional interactions between mitochondria and other cytoskeletal elements (Benard et al., 2007; Detmer & Chan, 2007; Romanello et al., 2010; Saks et al., 2010). This structural arrangement is lost during mitochondrial isolation procedures, and therefore, the assumption that mitochondria isolated from skeletal muscle should behave as they would in vivo is not a trivial concern.

In addition to disrupted structure upon isolation, most mitochondrial isolation methods yield 10–20% of the mitochondria within muscle (Kuznetsov et al., 2008; Figueiredo et al., 2009), which, because of isolation of specific sub-populations of mitochondria, may result in important bias (Piper et al., 1985;
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Kuznetsov et al., 2008). Finally, it has been suggested that mitochondria from aged muscles become larger and more fragile than their younger counterparts (Terman & Brunk, 2004), and depending upon how this impacts survival during isolation adds to concerns about how accurate and/or complete the representation of mitochondria with aging are following isolation methods (Tonkonogi et al., 2003; Figueiredo et al., 2008).

A more recent and routinely employed approach to study mitochondrial function in skeletal muscle involves gentle dissection and chemical permeabilization of the sarcolemma to produce permeabilized myofiber bundles. Unlike isolated mitochondrial preparations, this method permits representation of all mitochondria within a muscle fiber and preserves mitochondrial structural interactions and morphology (Kuznetsov et al., 2008). The only prior studies to use the permeabilized fiber approach in an aging context did not provide data concerning the degree of muscle atrophy (Tonkonogi et al., 2003; Hutter et al., 2007); no prior study has therefore applied this method to study mitochondrial function in a muscle where sarcopenia is well established.

Despite the concerns noted previously, to date, there has been no systematic comparison of mitochondrial function in aged muscles between isolated mitochondria and permeabilized myofiber bundles. To this end, we examined three primary indices of mitochondrial function in isolated mitochondria and saponin-permeabilized muscle fiber bundles from the mixed region of the gastrocnemius muscle in young adult (YA) and senescent (SEN) Fischer 344 × Brown Norway F1-hybrid (F344BN) rats: respiration, ROS emission, and mPTP sensitivity to Ca2+. These measures of mitochondrial function were chosen because they are relevant to the decreased maximal ATP-generating capacity (Hepple et al., 2004), increased oxidative damage accumulation (Mecocci et al., 1999; Fugere et al., 2006; Hepple et al., 2008), and increased activation of apoptosis (Alway et al., 2002; Dirks & Leeuwenburgh, 2002; Chabi et al., 2008) previously observed in aging muscles. Furthermore, the SEN age represents a stage where sarcopenia is well established in the F344BN rat model (Brown & Hass, 1996; Hagen et al., 2004; Seo et al., 2008), permitting relevant insight into the role of mitochondria in age-related muscle changes. Based upon the prevailing view that isolation procedures could selectively harvest the healthiest mitochondria (Tonkonogi et al., 2003; Kuznetsov et al., 2008), our a priori hypothesis was that isolated mitochondria would show less severe age-related impairments than permeabilized bundles.

Contrary to that hypothesis, we demonstrate that whereas isolated mitochondria from SEN muscle exhibit a marked reduction in respiratory capacity, higher H2O2 release under State III conditions, and increased mPTP sensitivity to Ca2+, in SEN-permeabilized fiber bundles, respiratory capacity is reduced only under complex IV-driven respiration, there is no difference in H2O2 emission, and there is a lesser change in mPTP sensitivity to Ca2+. In addition, our results indicate important qualitative differences in mitochondrial respiration and mPTP dynamics between YA- and SEN-isolated mitochondria, but not in permeabilized fiber bundles. As such, our findings indicate that routinely employed mitochondrial isolation procedures exaggerate functional age-related impairments in sarcopenic skeletal muscles. We conclude that the changes in mitochondrial function in a muscle where sarcopenia is well established are less severe than has typically been indicated by isolated organelle studies.

Results

Animal characteristics

The mass of the SEN animals (539 ± 30 g) was greater than that of the YA animals (400 ± 21 g; P < 0.05). On the other hand, gastrocnemius muscle mass was 38% less in SEN (1277 ± 36 mg) than YA (2054 ± 41 mg; P < 0.05), demonstrating an advanced state of sarcopenia.

Citrate synthase and complex IV activity in isolated mitochondria and fiber bundles

We measured mitochondrial protein yield in the mitochondrial isolation procedures, and citrate synthase (CS) and cytochrome c oxidase (COX) activities in both the isolated mitochondria and fiber bundles. Mitochondrial protein yield was very similar between age-groups (Fig. 1, inset). On the other hand, despite similar protein concentration of the isolated mitochondrial preparations in both age-groups, the CS (Fig. 1A) and COX (Fig. 1C) activities (normalized to ‘mitochondrial’ protein content) in isolated mitochondria were respectively 41% and 32% lower in the SEN age-group. These results from isolated mitochondria contrast with the very similar CS (Fig. 1B) and COX (Fig. 1D) activities between age-groups in the fiber bundle experiments. Note here that the data reported in Fig. 1D represent the activities measured in the fiber bundles used in H2O2 emission experiments and that similar results were obtained in the fiber bundles used in respirometry and Ca2+ retention capacity experiments (data not shown).

Characteristics of isolated mitochondria

For identical isolation procedures and matched protein concentration between age-groups, mitochondrial isolates from SEN muscle exhibited a markedly lower mitochondrial particle density, based on Mitotracker Red experiments (Fig. S1A,B). In fact, mitochondrial particle volume density (in μm3 mitochondrial particles mm−3) and fluorescence brightness density (in units of mean fluorescence intensity mm−2) were 44% and 60% lower in the SEN sample, respectively (data not shown). This difference in volume density is similar in magnitude to the mean age differences in CS and COX activity (Fig. 1A,C), whereas the difference in fluorescence density parallels mean age differences in respiration (Fig. 3A) and H2O2 emission (Fig. 4A) per mg of protein in isolated mitochondria experiments. In addition to these results indicating lower mitochondrial content in isolates from SEN muscle, protein content of representative subunits of complexes
I, II, III and IV was 47–69% lower in SEN vs. YA isolates, whereas protein content of subunit alpha of complex V was not different between YA and SEN isolates (Fig. 2). Median mitochondrial particle volume was greater in SEN (Fig. S1C), and median mitochondrial particle Mitotracker Red intensity was lower in SEN mitochondria (Fig. S1D).

Mitochondrial respiration

Representative raw tracings of respirometry experiments are shown in Fig. S2 (Supporting information). Respiratory control ratio (State III GM/GM) in both the isolated mitochondria and the permeabilized bundles showed the preparations to be of high quality (Fig. 3A,B insets) (Frezza et al., 2007b; Kuznetsov et al., 2008). Similar to the biochemical activities noted earlier, the isolated mitochondria from SEN animals exhibited rates of respiration that were 40–60% lower than in YA (Fig. 3A), whereas only N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)-supported respiration was lower in fiber bundles of SEN vs. YA (Fig. 3B). As expected, normalizing for COX activity eliminated the difference in TMPD-driven respiration (direct stimulation of complex IV), but the other differences between YA- and SEN-isolated mitochondria remained (Fig. 3C). Similarly, normalization to COX activity in fiber bundles eliminated

Fig. 1 Mitochondrial enzyme activity is lower with aging in isolated mitochondrial preparations but not in permeabilized fiber bundles. (A) Comparison of citrate synthase (CS) activity measured biochemically from gastrocnemius muscle isolated mitochondria of young adult (YA) and senescent (SEN) rats. The inset represents protein yield recovered from mitochondrial isolation procedures in YA and SEN. (B) Comparison of CS activity measured from permeabilized bundles of YA and SEN gastrocnemius muscles. (C) Comparison of complex IV activity (COX) measured biochemically from isolated mitochondria homogenates. (D) COX activity measured from permeabilized bundles. Enzyme activities for the fiber bundles are reported for the bundles used in H2O2 emission experiments and are similar to results obtained in bundles used for respirometry. *P < 0.05 vs. YA. COX, cytochrome c oxidase.

Fig. 2 Mitochondrial protein content is lower in senescent (SEN) mitochondrial isolates. (A) Comparison of the relative content of electron transport chain complexes subunits in young adult (YA)- and SEN-isolated mitochondria. Values are expressed relative to protein content of complex I in YA. (CI: complex I; CII: complex II, CIII: complex III; CV: complex IV; CV: complex V). (B) Representative Western blots of isolated mitochondria probed for subunits of each ETC. complex. *P < 0.05 vs. YA.
the difference in TMPD-driven respiration between YA and SEN (Fig. 3D).

Mitochondrial H$_2$O$_2$ emission

The results for H$_2$O$_2$ emission from isolated mitochondria were very similar to respiration results in that H$_2$O$_2$ emission was markedly lower in SEN than YA mitochondria for all states where substrates were present (Fig. 4A), whereas there were no detectable differences between age-groups in fiber bundles (Fig. 4B). Normalization for COX activity eliminated all differences between YA and SEN mitochondria, except H$_2$O$_2$ emission with succinate and under conditions of complex III blockade by antimycin A (AA), which remained lower in the SEN mitochondria (Fig. 4C). On the other hand, normalization for COX activity had no impact on the lack of differences in H$_2$O$_2$ emission between age-groups in fiber bundles (Fig. 4D). When expressed per O$_2$ flux, H$_2$O$_2$ release in SEN-isolated mitochondria was similar to YA under basal and glutamate-malate conditions, but was 58% greater under State III respiration compared to YA (Fig. 4E). There was no significant age difference in H$_2$O$_2$ release after normalizing for O$_2$ flux in permeabilized bundles (Fig. 4F). In isolated mitochondria, blocking electron flow at complex III with AA following State III conditions resulted in a 39-fold increase in H$_2$O$_2$ release per unit of O$_2$ flux in YA, compared to a 15-fold increase in SEN. In permeabilized bundles, complex III blockade following State III conditions resulted in a 20-fold increase in YA, compared to an 11-fold increase in SEN. Despite these differences in the extent of increase in H$_2$O$_2$ per unit of O$_2$ flux under these conditions, this did not result in a significant difference in net H$_2$O$_2$ emission per unit of O$_2$ flux between YA and SEN groups in either preparation following AA treatment.

Mitochondrial calcium retention capacity

Isolated mitochondria from SEN exhibited a 39% lower Ca$^{2+}$ retention capacity (CRC) vs. YA mitochondria (Fig. 5A), whereas
there were no differences between age-groups in phantom bundles (Fig. 5B). Normalization for COX activity abolished these differences between YA- and SEN-isolated mitochondria (Fig. 5C) and had no impact on the lack of differences between age-groups in fiber bundles (Fig. 5D).

**Time to opening of mPTP**

Representative traces of Ca$^{2+}$ uptake and release in isolated mitochondria and phantom fiber bundles of YA (Fig. 6A) and SEN (Fig. 6B) show a significantly shorter time to pore opening.
in isolated mitochondria (10–20 s) compared to bundles (650–1000 s). In both isolated mitochondria and bundles, there was a shorter time to pore opening in SEN. The mean values of these experiments revealed a 47% shorter time to pore opening in SEN- vs. YA-isolated mitochondria (Fig. 6C) compared to a 29% shorter time to pore opening in SEN vs. YA in phantom bundles (Fig. 6D).

**Respiratory and biochemical activity ratios**

The integrity of the electron transport chain is reflected in the stoichiometry of respiration rates driven by the different mitochondrial complexes. The ratios of several steps during respiration experiments in isolated mitochondria revealed marked differences in the functional qualities of the mitochondria isolated from SEN vs. YA (Fig. 7A). Specifically, in isolated mitochondria from SEN, we demonstrate higher complex I + II (succinate step) and IV respiration (TMPD step) relative to complex I alone [adenosine di-phosphate (ADP) step; 109% and 168%, respectively] and higher complex IV respiration relative to complex I + II (155%). Similarly, the ratio of COX and CS biochemical activities in SEN was 116% of that of YA. Consistent with many of the other comparisons between isolated mitochondria and fiber bundles, there were no differences in the respiratory or biochemical activity ratios between ages in the fiber bundles (Fig. 7B). Collectively, the respiratory and biochemical activity ratios in isolated mitochondria are consistent with a preferential loss of matrix constituents in the SEN age-group during the isolation process.

**Discussion**

This study provides the first systematic assessment of mitochondrial function in sarcopenic skeletal muscle using two commonly used approaches in parallel: isolated mitochondria (*in vitro*) and permeabilized bundles (*in situ*). Among the differences between these approaches is that whereas mitochondrial isolation disrupts normal mitochondrial interactions and morphology and yields only a fraction of all mitochondria in intact muscle, the permeabilized bundle technique preserves mitochondrial interactions and morphology and provides representation of the totality of the mitochondrial population within muscle (Kuznetsov et al., 2008; Saks et al., 2010). It was on the basis of these differences that we hypothesized that there would be less severe age-related impairments in isolated mitochondria than in fiber bundles because of selective harvest of healthiest mitochondria in isolation procedures. On the contrary, we found that age-related changes were much more severe in isolated mitochondria than permeabilized bundles. In seeking to explain this result, we note that the changes observed with aging in isolated mitochondria were very similar to previous studies showing reduced mitochondrial respiratory capacity (Desai et al., 1996;
Drew et al., 2003; Short et al., 2005), increased ROS generation under some conditions (Capel et al., 2004; Vasilaki et al., 2006; Muller et al., 2007; Chabi et al., 2008), and increased susceptibility of mPTP opening (Chabi et al., 2008; Seo et al., 2008) in mitochondria isolated from aged muscles. On the other hand, we observed relatively mild effects of aging on these indices of mitochondrial function in permeabilized myofiber bundles, and it is this novel observation that sets our results apart from previous studies. It is also important to note that because we used a protease (Nagarse) during our isolation methods, the resulting isolates contain both subsarcolemmal and intermyofibrillar mitochondria (Lanza & Nair, 2009), facilitating comparisons to permeabilized bundles where both mitochondrial populations are present. We conclude that routine mitochondrial isolation procedures yield a markedly exaggerated perception of mitochondrial dysfunction in aged sarcopenic muscle and that mitochondrial functional alterations in aged muscles are much less severe than have been typically considered.

**Mitochondrial content in isolated mitochondrial preparations**

We used CS activity, a mitochondrial matrix enzyme and recognized marker of mitochondrial volume (Schwerzmann et al., 1989; Picard et al., 2008), and COX activity, an inner mitochondrial membrane-embedded enzyme, to provide estimates of mitochondrial content in our isolated mitochondrial preparations. Our results suggest significantly lower mitochondrial content in SEN- than in YA-isolated mitochondrial preparations. Because CS and COX activities in permeabilized bundles were similar between YA and SEN (Fig. 1B), these results indicate that a lower proportion of mitochondria were extracted from sarcopenic aged muscles despite identical isolation procedures. Consistent with this interpretation, we observed lower absolute amounts of mitochondrial particles in the SEN group with high-resolution confocal microscopy. Efficiency of mitochondrial extraction during the isolation process may be influenced by differences in collagen deposition and connective tissue composition with aging (Goldspink et al., 1994) and/or by the presence of more fragile mitochondrial structures in aged muscles (Piper et al., 1985; Terman & Brunk, 2004). Another possibility is that similar amounts of mitochondrial protein were in fact extracted from the aged muscles during isolation but that a high proportion of these were unable to reconstitute into functional mitochondria and were therefore unable to develop the necessary membrane potential to take up the Mitotracker Red compound. In relation to this point, we note that the amounts of representative subunits of complexes I, II, III and IV detected by Western
Mitochondrial respiration, H$_2$O$_2$ release and sensitivity to mPTP opening

In our study, we chose to examine three indices of mitochondrial function that have relevance to sarcopenia. Specifically, we examined respiratory capacity, H$_2$O$_2$ emission, and the sensitivity of the mPTP pore to a Ca$^{2+}$ challenge. We chose these measures because they relate to the decrease in muscle aerobic capacity (Conley et al., 2000; Hagen et al., 2004; Short et al., 2005), increased oxidative damage (Mecocci et al., 1999; Cao et al., 2001; Aiken et al., 2002; Bua et al., 2002; McKenzie et al., 2002; Mansouri et al., 2006), and increased apoptosis (Strasser et al., 2000; Dirks & Leeuwenburgh, 2002; Rice & Blough, 2006) frequently reported in aged skeletal muscles. Whereas our results in isolated mitochondria are similar to many previous studies showing an apparent reduction in respiratory capacity per mitochondrion in aged muscles (Trounce et al., 1989; Kumaran et al., 2005; Chabi et al., 2008), our results in permeabilized myofibers show only a small reduction in complex IV-driven respiration with aging (Fig. 4). Interestingly, normalization of respiration in isolated mitochondrial preparations to COX activity brought the SEN values closer to YA, consistent with the idea that some of the difference we observed between YA- and SEN-isolated mitochondrial preparations was because of lower mitochondrial content in the isolates yielded from SEN muscle. However, age-related differences still remained in the isolates that were not seen in the permeabilized bundles, which we conclude must be an artifact of the isolation procedure. In this respect, our confocal imaging results showed lower mean fluorescence intensity per mitochondrial particle in SEN mitochondria. As the Mitotracker probe is taken up in proportion to mitochondrial content, our results suggest the lower respiratory capacity of SEN-isolated mitochondria after normalizing for COX activity may be related to a lower membrane potential. Further to this point, we also note that the median mitochondrial particle size was greater in SEN isolates, suggesting greater swelling than is typically associated with isolation procedures (Schwerzmann et al., 1989; Figueiredo et al., 2002) frequently reported in aged skeletal muscles.

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![Diagram](image_url)

Fig. 7 Respiratory and biochemical activity ratios are altered with aging in isolated mitochondria but not in permeabilized bundles. (A) Comparison of the relative respiratory rates (O$_2$ flux) measured in isolated gastrocnemius muscle mitochondria of young adult (YA) and senescent (SEN) rats. Oxygen flux was induced by selective activation of complexes of the electron transport chain with GM + ADP (I), GMS + ADP (I + II) and TMPD/ascorbate (IV). Ratios of the respective respiratory activities are shown. Relative biochemical activity of cytochrome c oxidase (COX) (complex IV) and citrate synthase (CS) is also shown. (B) Relative respiratory rates (I + II/I; IV/I; IV/I + II) and biochemical activity (COX/CS) measured in permeabilized bundles of YA and SEN gastrocnemius muscles. *P < 0.05 vs. YA. AA, adenosine di-phosphate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

Mitochondrial respiration and electron transport within the mitochondria are associated with ROS production and H$_2$O$_2$ release (Stowe & Camara, 2009), which not only trigger important signaling pathways but can also cause molecular damage and lead to nuclear apoptosis at high levels (St-Pierre et al., 2006; Stowe & Camara, 2009). When expressed per unit O$_2$ flux, we find that H$_2$O$_2$ release from isolated mitochondria is greater in SEN under conditions of maximal respiration (State III, GMS), but we find no significant differences in H$_2$O$_2$ release between YA and SEN in permeabilized bundles. Therefore, although our data in isolated mitochondria are consistent with several reports indicating higher ROS production from aged mitochondria (Capel et al., 2004; Mansouri et al., 2006; Chabi et al., 2008), our data suggest that this effect of aging may be limited to, or at least exaggerated by, the mitochondrial isolation...
approach. On the other hand, we note that the $\text{H}_2\text{O}_2$ emission we report reflects the combined effects of superoxide production, the rate of its conversion to $\text{H}_2\text{O}_2$ by superoxide dismutase, and finally, the binding of $\text{H}_2\text{O}_2$ to the Amplex red probe to form resorufin. In this respect, recent work indicates that glutathione peroxidases compete with the Amplex Red probe and therefore leads to an underestimate of superoxide production via this method (Treberg et al., 2010). As we have previously reported a doubling of glutathione peroxidase activity in vastus lateralis muscle of SEN F344BN rats previously (Thomas et al., 2010), which is a muscle of similar fiber type to the gastrocnemius muscle (Armstrong & Phelps, 1984), the magnitude of any increase in mitochondrial ROS production with aging would be concealed by an up-regulation of glutathione peroxidase or other antioxidant enzymes. Furthermore, if the amount of glutathione peroxidase enzyme, which is located in the mitochondrial matrix, is reduced as a result of the mitochondrial isolation procedure, this could account for the differences in $\text{H}_2\text{O}_2$ emission seen with aging between isolated mitochondria and permeabilized bundles.

Because apoptosis has been strongly implicated in sarcopenia (Siu, 2009), with many studies showing evidence of increased apoptotic activation in aged muscle (Dirks & Leeuwenburgh, 2002; Phillips & Leeuwenburgh, 2005; Siu et al., 2005; Baker & Hepple, 2006; Rice & Blough, 2006), we investigated one of the most potent triggers for apoptosis: mitochondrial outer membrane permeabilization. Physiologically, mitochondrial outer membrane permeabilization is triggered by mPTP opening and is an irreversible event associated with the release of pro-apoptotic factors by the mitochondria (Rasola & Bernardi, 2007). Our result showing an increased sensitivity of mPTP opening to a $\text{Ca}^{2+}$ challenge in isolated mitochondria with aging is consistent with recent data using the same animal model and ages as studied here (Chabi et al., 2008; Seo et al., 2008).

In our study, mitochondrial outer membrane permeabilization kinetics assessed by time to mPTP opening demonstrate a more pronounced increase in mPTP sensitivity with age in isolated mitochondria than in permeabilized bundles, leading to an exaggerated impression of susceptibility to apoptosis in aged isolated mitochondria. Furthermore, isolated mitochondria from both YA and SEN groups display a dramatic hypersensitivity to $\text{Ca}^{2+}$ compared to permeabilized bundles. As under physiologic conditions mitochondrial outer membrane permeabilization and apoptotic signaling is enhanced by mitochondrial fragmentation (Arnoult, 2007; Detmer & Chan, 2007; Ong et al., 2010) and prevented by mitochondrial fusion (Frezza et al., 2007a), differences in mPTP sensitivity between preparations may be secondary to mechanical fragmentation of mitochondria during isolation. Consistent with this premise, we demonstrate that mitochondrial isolation mechanically fragments mitochondria into small spherical particles, that this phenomenon is associated with marked sensitization of isolated organelles to mPTP opening compared to permeabilized bundles, and that this sensitization effect is more pronounced in the SEN-isolated mitochondria. As such, the indices of respiratory capacity and ROS production, our results in permeabilized bundles indicate less severe alterations in the function of the mPTP in aging muscle than has been previously suggested.

**Qualitative differences between isolated mitochondria and permeabilized bundles**

An important consideration in the present study relates to the preferential loss of CS (soluble in matrix) compared to COX (inner mitochondrial membrane-anchored) with aging in the isolated mitochondrial preparation (41% vs. 29% lower in SEN, respectively). This suggests that the marked impairment of mitochondrial respiration and altered $\text{H}_2\text{O}_2$ release with aging in isolated mitochondria but not permeabilized bundles may be in part because of a selective loss of mitochondrial matrix constituents during the isolation process owing to transient rupture/resealing of the outer and inner mitochondrial membranes (Schwerzmann et al., 1989), particularly in the aged mitochondrial preparations. Selective loss of matrix enzymes (Krebs cycle enzymes) and metabolic intermediates (nicotinamide adenine dinucleotide, NAD+; flavin adenine dinucleotide, FAD+) essential for complex I and complex II respiration or antioxidant capacity (e.g., glutathione peroxidase) would certainly influence results from respiration and $\text{H}_2\text{O}_2$ assays, and possibly other aspects of mitochondrial function.

To provide insight into this possibility, mitochondrial respiration was sequentially stimulated to allow assessment of the relative activity of different complexes of the electron transport chain. Notably, complex IV activity was directly stimulated by TMPD and therefore independent of mitochondrial matrix enzymes and of electron transport by upstream complexes. In SEN- vs. YA-isolated mitochondria only, we demonstrate higher complex IV activity relative to complex I and complexes I + II operating together. As mentioned earlier, loss of matrix enzymes necessary to convert substrates for mitochondrial respiration and produce reducing equivalents (NADH, $\text{FADH}_2$) would selectively reduce complex I- and II-driven respiration. Moreover, this isolation effect is exaggerated in SEN-isolated mitochondria, suggesting that mitochondria from SEN muscles are more likely to lose matrix content. Also, note that the relative amount of subunits from complex I and complex II was proportionally greater than complex IV in SEN isolates when probed by Western blot, showing that this altered respiratory stoichiometry is not because of lower protein levels of complexes I and II in SEN isolates. Collectively, our findings indicate that mitochondrial isolation procedures induce specific alterations of mitochondrial respiration in aged muscle mitochondria that are not seen in situ and that this effect is likely in part because of greater loss of matrix constituents during isolation in SEN mitochondria.

**Conclusions**

Taken together, our findings clearly establish that isolated muscle mitochondria exhibit exaggerated impairments with aging
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when compared with permeabilized muscle bundles. Although some of this effect is attributable to differences in mitochondrial content between YA- and SEN-isolated mitochondrial preparations, we also show important qualitative differences in mitochondrial morphology and function, which are independent of mitochondrial content. In contrast, our results in permeabilized myofibers reveal only a minor defect in mitochondrial respiratory capacity, no change in ROS emission (although an increase in glutathione peroxidase activity with aging likely conceals an increase in ROS), and a mild increase in sensitivity of the mPTP to opening in SEN skeletal muscle. As such, our results indicate that changes in mitochondrial function in aged sarcopenic skeletal muscle are less severe than typically indicated from isolated organelle studies.

One interpretation of our results is that mitochondrial isolation may reveal weaknesses within the mitochondrial machinery that are present but not evident in vivo or in permeabilized bundles (in situ). This warrants caution in directly translating putative weaknesses observed in isolated mitochondria to physiologically relevant dysfunction in vivo. On the other hand, these in vitro preparations may nevertheless be useful in the context of aging to study key aspects of mitochondrial biology given further understanding of how the isolation procedures affect mitochondrial structure and composition. In particular, we suggest that the isolation of mitochondria represents an intervention that can be used to facilitate the study of mitochondrial resilience and stress resistance, whereas the permeabilized bundle method is better suited to provide insight into the day-to-day function of mitochondria. On this basis, while our results in situ suggest mitochondrial function is relatively well preserved in sarcopenic muscle, our results also demonstrate that aged mitochondria have a markedly impaired ability to tolerate the stress of isolation, which may provide physiologically relevant insight into the ability of aged mitochondria to tolerate stress in general.

Experimental procedures

Animals and surgical methods

All procedures were conducted with approval from the University of Calgary Animal Care Committee. Male Fischer 344 × Brown Norway F1-hybrid (F344BN) rats were obtained from the colony maintained by the National Institute on Aging at ages of 8–10 months (YA) and 35–36 months (SEN). The ages were chosen to represent a period where there is substantial age-related muscle atrophy and dysfunction (Brown & Hasser, 1996; Hagen et al., 2004; Hepple et al., 2004) and thus to permit insight into changes that would be relevant to sarcopenia. Upon arrival at our institution, rats were housed individually in cages fitted with filter bonnets at the University of Calgary Biological Sciences vivarium and were kept a minimum of 48 h prior to being used in experiments (12:12 h light/dark cycle, ambient temperature 23 °C). Food and water were provided ad libitum.

On the day of experiment, rats were anesthetized with 55–65 mg kg⁻¹ sodium pentobarbital (i.p.). The left and right gastrocnemius (Gas) muscles from eight YA and eight SEN animals were carefully dissected and placed into ice-cold stabilizing Buffer A [in mM: 2.77 K₂C₅O₇, Ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetraacetic acid (EGTA), 7.23 K₃EGTA, 6.56 MgCl₂, 0.5 dithiothreitol (DTT), 50 2-(N-morpholino)ethanesulfonic acid potassium salt (K-MES), 20 imidazol, 20 taurine, 5.3 Na₂ATP, 15 phosphocreatine, pH 7.3 at 4 °C]. To facilitate greater homogeneity between samples obtained from a given Gas muscle, the highly oxidative red region and highly glycolytic white region of this muscle were removed to leave the mixed region of this muscle. The mixed Gas was then divided equally for mitochondrial isolation and permeobilized myofiber preparations.

Mitochondrial isolation

Mitochondrial isolation was performed using standard homogenization, protease digestion and differential centrifugation methods, similar to those described by (Frezza et al., 2007b). Mixed gas was weighed and placed in 20 mL of ice-cold mitochondrial extraction buffer (in mM: 100 sucrose, 50 KCl, 5 EDTA, 2 K₂HPO₄, 50 Tris-base, pH 7.4 at 4 °C) and subsequently minced manually with fine scissors. All steps were performed at 4 °C. Minced tissue was homogenized at 600 rpm with a motor-driven Teflon Potter Elvehjem pestle (Corning Inc., Lowell, MA, USA) (six up and down pulses), incubated with 1 mg g⁻¹ Nagarse protease (P8038; Sigma-Aldrich, Oakville, ON, Canada) for 5 min, diluted further with another 20 mL extraction buffer and homogenized again at 600 rpm (four up and down pulses). The homogenate was centrifuged at 1000 g for 10 min, after which the mitochondria-rich supernatant was filtered through cheesecloth and the pellet discarded. Mitochondria were then pelleted by centrifugation at 8000 g for 10 min and gently re-suspended in re-suspension buffer (in mM: 100 sucrose, 50 KCl, 0.05 EDTA, 2 K₂HPO₄, 50 Tris-base, pH 7.4 at 4 °C), centrifuged again at 8000 g for 10 min, and the final pellet gently re-suspended in 600 µL of re-suspension buffer. Mitochondrial protein concentration was measured spectrophotometrically using the bicinchoninic acid assay (23225; Thermo Scientific, Waltham, MA, USA). Isolated mitochondria were used fresh for functional measurements. A portion of fresh isolated mitochondria was frozen for Western blots and enzymatic activity measurements.

Preparation of permeabilized myofiber bundles

Dissection and permeabilization of myofiber bundles with saponin was performed according to methods described by Kuznetsov et al. (Kuznetsov et al., 2008) and as we have described previously (Picard et al., 2008). After dissection, muscles were immediately put on ice in precooled (4 °C) Buffer A (described previously) and weighed. Whole muscles were trimmed of connective tissue and manually teased into small fiber bundles. Once dissection was completed, fiber bundles were placed in
Buffer A supplemented with 0.05 mg mL\(^{-1}\) saponin to allow selective permeabilization of the sarcolemma. Following 30 min of incubation at low rocking speed, fiber bundles were subjected to 3 × 10 min rinses in Buffer B (in mM: 2.77 Ca\(_2\) EGTA, 7.23 K\(_2\) EGTA, 1.38 MgCl\(_2\), 3.0 K\(_2\)HPO\(_4\), 0.5 DTT, 20 imidazole, 100 K-MES, 20 taurine, pH 7.3, at 4 °C) supplemented with fatty acid-free bovine serum albumin (BSA: 2 mg mL\(^{-1}\)). Fiber bundles for respiration experiments were kept in Buffer B on ice until use.

**High-resolution respirometry**

Permeabilized myofiber and isolated mitochondrial respiration were assessed with a polarographic oxygen sensor (Oxygraph-2k, Oroboros, Innsbruck, Austria) calibrated as required for O\(_2\) concentration, environmental variables, and auto O\(_2\) consumption. Briefly, 3.5–6 mg (wet weight) permeabilized bundles or 0.01 mg mitochondrial protein, prepared as described earlier, was added to 2 mL of Buffer B in the respirometer and equilibrated for baseline endogenous respiration at 37 °C. Myofiber respiration was performed at hyperoxygenated levels to eliminate O\(_2\) diffusion limitations. The substrate protocol assessing O\(_2\) flux was added sequentially as follows, with each step interspersed with a period of stabilization between injections: 10 mM glutamate + 2 mM malate (GM), 2 mM ADP, 10 μM succinate (Succ), 10 μM cytochrome c, 10 μM AA, 5 mM ascorbate + 0.5 mM TMPD. To account for TMPD auto-oxidation, rates of TMPD oxidation were initially determined at different O\(_2\) concentrations without samples present. Auto-oxidation-associated respiration was then subtracted from TMPD respiration values obtained in the presence of samples. After respiration measurements were completed, bundles were removed and placed in liquid N\(_2\) and stored at −80 °C for enzymatic measures. Respiration was expressed as picomoles per second per mg wet weight for bundles, per mg protein for isolated mitochondria, and as nanomoles per enzymatic unit (U) of cytochrome oxidase activity for both preparations.

**ROS emission**

Reactive oxygen species emission was detected by measuring the rate of appearance of resorufin from Amplex Red with a F-2500 fluorescence spectrophotometer at an excitation/emission wavelength of 563/587 nm, using the FL solutions software. Resorufin is formed by the 1:1 reaction of H\(_2\)O\(_2\) and Amplex Red and is catalyzed by horseradish peroxidase. A standard curve was generated daily from the slope of \(\Delta F/\text{min}\) under experimental conditions in the absence of samples and used to calculate the rate of H\(_2\)O\(_2\) production. Samples were prepared as described earlier. Permeabilized bundles used for ROS measurement were further washed 3 × 10 min in Buffer Z (in mM: 110 K-MES, 35 KCl, 1 EGTA, 3 MgCl\(_2\), 10 K\(_2\)HPO\(_4\), pH 7.3 at 4 °C) supplemented with BSA (5 mg mL\(^{-1}\)). Bundles (4–6 mg wet weight) or isolated mitochondria (0.01 mg) were added to a thermostatted, magnetically stirred cuvette containing 600 μL Buffer Z, Amplex Red (5.5 μM), and horseradish peroxidase (1 U mL\(^{-1}\)), after a period of baseline autofluorescence. All measures were performed at 37 °C. After the reaction was initiated, substrates were added as follows (allowing a period of stabilization between each step): GM (10 + 2 mM), Succ (10 mM), ADP (10 μM), ADP (100 μM), ADP (1 mM), AA (10 μM). At the conclusion of the ROS measurements, bundles were placed in liquid N\(_2\) and stored at −80 °C for enzymatic analysis.

H\(_2\)O\(_2\) emission is expressed as picomoles per minute per mg wet weight for bundles, per mg protein for isolated mitochondria, and as per U of cytochrome oxidase for both preparations.

**Biochemical assays for CS and COX**

Citrate synthase and COX activity were used as representative of a mitochondrial matrix enzyme and an electron transport chain enzyme, respectively, and to estimate mitochondrial content in each preparation (Schwerzmann et al., 1989). For these measurements, frozen permeabilized bundles used for respirometry and ROS emission assays, and isolated mitochondria frozen immediately after isolation were used. All samples were homogenized in an extraction buffer containing 50 mM triethanolamine and 1 mM EDTA. Permeabilized bundles were finely minced using small scissors and homogenized on ice using a small pestle rotor in 1:20 w/v. Isolated mitochondria were diluted 1:10 w/v, vigorously vortexed, and incubated on ice for 20 min. Citrate synthase activity was measured spectrophotometrically by detecting the increase in absorbance at 412 nm in a 96-well plate at 30 °C, using 200 μL of a reaction buffer (200 mM Tris, pH 7.4) containing (in mM: 2 acetyl-CoA, 200 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 350 oxaloacetic acid, 0.1% Triton-x). Cytochrome c oxidase activity was measured by detecting the decrease in absorbance at 550 nm in a 96-well plate at 30 °C, using 200 μL of a reaction buffer (potassium phosphate 100 mM, pH 7.0) containing 0.1% n-dodecylmaltoside and 0.1 mM purified reduced cytochrome c. The molar extinction coefficients used were 13.6 L mol\(^{-1}\) cm\(^{-1}\) for DTNB and 29.5 L mol\(^{-1}\) cm\(^{-1}\) for reduced cytochrome c.

**Imaging of isolated mitochondria**

Freshly isolated mitochondria were diluted to a protein concentration of about 2.5 mg mL\(^{-1}\) (see Fig. 2 for specific values) and incubated with 16.7 μM of Mitotracker Red CMXROS (Molecular Probes M7512, Invitrogen Canada Inc., Burlington, ON, Canada) for 20 min at 30 °C. Ten microliters of labeled mitochondria were placed on a glass slide and mounted with a coverslip to be imaged. Excess liquid was extruded, mitochondria were left to settle for 5 min, and images were acquired using a confocal microscope (Olympus Fluoview FV1000, Olympus Fluoview version 2.0c software, Olympus Canada, Markham, ON, Canada) with a PlanApo N 60x/1.42 oil-immersion objective and 1.6 digital zoom (96× final, 1 pixel = 0.0827 μm). Alexa Fluor 546 (Invitrogen Canada Inc., Burlington, ON, Canada) excitation settings
were used with pinhole size of 110 μm, z-slices of 0.5 or 0.3 μm, and the following laser settings: high voltage (HV) = 369, Gain = 1, Offset = 21. Imaris 7.0 software was used to analyze z-stacks and produce surface renderings, volume and mean fluorescence intensity measurements. Software settings were smooth deactivated; diameter of largest sphere of 0.7 μm; threshold for background subtraction of 1030 μm²; split touching objects enabled; estimated diameter of 0.444 μm; quality threshold above 260; and sphericity threshold above 0.550 (94%+ selection). Representative images were obtained from the microscope’s natural perspective (top view) and from the perpendicular perspective (side view) of the 3D surface analysis. Volume and mean fluorescence intensity (independent of particle size) were computed for each individual particle.

### Western blotting for electron transport chain composition in isolated mitochondria

Frozen–thawed mitochondrial isolates were used in Western blotting experiments to determine the relative amounts of each of the electron transport chain complexes in each age-group. Briefly, 5 μg of protein was loaded from each isolate into precast 4–15% SDS–polyacrylamide gels (SDS–PAGE) (Bio-Rad, Hercules, CA, USA) and ran for 1.5 h at 110 V. Proteins were then electro-transferred for 1.5 h at 400 mA onto a polyvinylfluoride membrane (PVDF) and incubated overnight with a premixed cocktail of polyclonal antibodies directed against representative subunits of each of the electron transport chain complexes (Mitosciences MS604, 6 μg mL⁻¹, Mitosciences, Eugene, OR, USA). Equal protein loading was verified using the Ponceau red stain. Membranes were washed in 0.05% Tween–PBS buffer and incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:1000). Signals were detected using the enhanced chemiluminescence detection system (Pierce), and chemiluminescence was digitally captured (Syngene Bio-Imager, Frederick, MD, USA) and densitometry measured using the Bio-imager software (Syngene Tools).

### Preparation of phantom fiber bundles

In normal permeabilized myofibers, myosin/actin-associated proteins with high affinity for Ca²⁺ prevent measurements of mitochondrial calcium uptake (Saks et al., 1998; Picard et al., 2008). We therefore prepared phantom fibers without myosin as previously described (Saks et al., 1998). Fiber bundles were first permeabilized with saponin and washed three times in Buffer B as described earlier, then washed three times for 10 min in Buffer C (in mM: K-MES 80, HEPES 50, taurine 20, DTT 0.5, MgCl₂ 10, ATP 10, pH 7.3 at 4 °C). Fibers where then incubated for 30 min with intermittent manual agitation at 4 °C. A drop in signal to account for intrinsic Ca²⁺ buffering capacity of myosin but which preserves mitochondrial function (Picard et al., 2008).

### Statistical analyses

All values are presented as means ± standard error (SEM). Two-tailed student’s T test assuming unequal variance was used to determine P values. P value = 0.05 was considered significant. To account for unequal sample size in the analysis of isolated mitochondria, a Mann-Whitney rank sum test was used to determine P values.

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Author contributions

RTH and MP designed the experiments, which were performed by MP, DR, and KJW. Animal surgery was performed by MMT and assisted by SLR. CR ran the Western blots. The data were analyzed by MP, RTH, and DR. The confocal imaging experiments and analysis were carried out by MP. RTH and MP interpreted the data, and the manuscript was written by RTH, MP, TT, and DR.

References


atrophy involve a common program of changes in gene expression. FASEB J. 18, 39–51.


Supporting Information
Additional supporting information may be found in the online version of this article:

Fig. S1 Confocal imaging shows lower mitochondrial content in SEN isolated mitochondrial preparations.
Fig. S2 Representative traces of mitochondrial respiration.

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Supplementary Figure 1

Figure S1. Confocal imaging shows lower mitochondrial content in SEN isolated mitochondrial preparations. (A) (Upper) Top and (Lower) side views of 3-dimensional (3D) reconstruction from confocal microscope images of Gastrocnemius muscle isolated mitochondria from a YA rat. (B) (Upper) Top and (Lower) side views of 3D reconstruction from confocal microscope images of gastrocnemius isolated mitochondria isolated from a SEN rat with identical conditions as in A. Protein concentration was similar between these samples: 2.62 mg ml-1 in YA and 2.27 mg ml-1 in the SEN sample. (C) Comparison of mitochondrial particle volume measured from the YA and SEN preparations. (D) Comparison of mean particle fluorescence intensity measured based on the mitochondrial membrane potential-dependent incorporation of Mitotracker dye in YA and SEN preparations. Samples used for these experiments contained 15,490 particles for YA and 5,330 particles for SEN. Values are medians ± s.e.m. *P<0.05 versus YA.
**Supplementary Figure 2**

**Figure S2.** **Representative traces of mitochondrial respiration.** Shown are traces of (A) YA isolated mitochondria, (B) SEN isolated mitochondria, (C) YA permeabilized bundles, (D) SEN permeabilized bundles.

*mitos*: addition of 0.01mg of mitochondrial proteins or of a 4-6mg wet weight permeabilized fiber bundle; *GM*: glutamate (10mM) + malate (2mM); *ADP*: adenosine diphosphate (2mM); *SUCC*: succinate (10mM); *Cytc*: cytochrome c (10µM); *AA*: antimycin A (10µM); *TMPD*: TMPD (0.5mM) + ascorbate (5mM).