Age-Associated Impairments in Mitochondrial ADP Sensitivity Contribute to Redox Stress in Senescent Human Skeletal Muscle

Highlights

- Simultaneous measurements of mitochondrial respiration and ROS production in muscle
- Mitochondrial ADP sensitivity is decreased in skeletal muscle of older individuals
- Reduced ADP sensitivity increases mitochondrial ROS in older individuals
- Resistance-type exercise improves muscle health but does not recover redox balance

In Brief

Holloway et al. show that an inability of ADP to decrease mitochondrial reactive oxygen species emission contributes to redox stress in skeletal muscle tissue of older individuals and that this process is not recovered following prolonged resistance-type exercise training, despite the general benefits of resistance training for muscle health.
Age-Associated Impairments in Mitochondrial ADP Sensitivity Contribute to Redox Stress in Senescent Human Skeletal Muscle

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https://doi.org/10.1016/j.celrep.2018.02.069

SUMMARY

It remains unknown if mitochondrial bioenergetics are altered with aging in humans. We established an in vitro method to simultaneously determine mitochondrial respiration and H2O2 emission in skeletal muscle tissue across a range of biologically relevant ADP concentrations. Using this approach, we provide evidence that, although the capacity for mitochondrial H2O2 emission is not increased with aging, mitochondrial ADP sensitivity is impaired. This resulted in an increase in mitochondrial H2O2 and the fraction of electron leak to H2O2, in the presence of virtually all ADP concentrations examined. Moreover, although prolonged resistance training in older individuals increased muscle mass, strength, and maximal mitochondrial respiration, exercise training did not alter H2O2 emission rates in the presence of ADP, the fraction of electron leak to H2O2, or the redox state of the muscle. These data establish that a reduction in mitochondrial ADP sensitivity increases mitochondrial H2O2 emission and contributes to age-associated redox stress.

INTRODUCTION

Aging is a complex process associated with skeletal muscle and strength loss as well as insulin resistance (Baumgartner et al., 1998; Nair, 2005). The cellular mechanisms causing muscular and/or metabolic dysfunction with aging remain poorly understood. However, one proposed mechanism of action driving the aging process is an increase in mitochondrial-derived reactive oxygen species (ROS) (Jang et al., 2010). Specifically, increased ROS emission has been associated with motor unit loss and abnormal morphology (Kuwahara et al., 2010), muscle fiber atrophy (Kondo et al., 1991; Min et al., 2011), insulin resistance (Houstis et al., 2006), inflammation (Khodr and Khalil, 2001), and apoptosis (Simon et al., 2000). Conversely, transgenic and pharmacological approaches that attenuate mitochondrial ROS have been shown to preserve insulin sensitivity (Anderson et al., 2009), mitochondrial content (Lee et al., 2010), and muscle mass in diverse models (Min et al., 2011; Siegel et al., 2013) while also prolonging lifespan (Schriner et al., 2005). Altogether, these data implicate mitochondrial ROS as a fundamental mechanism of action influencing the aging phenotype.

Although these elegant rodent models provide compelling evidence to link mitochondrial ROS with age-associated pathologies, the data in humans remain ambiguous. For instance, although indices of redox stress have repeatedly been shown to increase with human aging (Barrientos et al., 1997; Rizvi and Maurya, 2007; Sohal et al., 2002), in vitro assessments of mitochondrial ROS emission do not appear to increase (Capel et al., 2005; Distefano et al., 2017; Gouspillou et al., 2014; Gram et al., 2015). These contradictory findings suggest that either mitochondria are not responsible for the increased oxidative stress with aging or, alternatively, contemporary in vitro assessment of mitochondrial ROS emission does not accurately reflect in vivo responses. In this respect, rates of ROS emission have almost exclusively been determined in the presence of succinate, and the subsequent reverse flow of electrons from complex II to complex I, which represents the capacity of mitochondria to produce ROS. However, stoichiometrically, succinate represents a small percentage of the electrons entering the electron transport chain (ETC; ~17%), and the delivery of electrons to the FMN binding site of complex I promotes forward electron flow, which has been shown to dramatically attenuate succinate-mediated ROS formation (Lambert and Brand, 2004). In addition, previous assessments of mitochondrial ROS with aging have been conducted in the absence of ADP (usually with oligomycin). However, the movement of ADP into the matrix, and the subsequent binding of ADP to the F1 subunit of ATP synthase, decreases proton motive force and the production of mitochondrial ROS (Anderson and Neuffer, 2006; Anderson et al., 2007; Picard et al., 2010). Importantly, resting skeletal muscle has ~25–100 μM free ADP (Perry et al., 2008; Phillips et al., 1996), and ADP transport is a highly regulated process that is attenuated with rodent models of insulin resistance (Smith et al., 2013) and improved following high-intensity exercise (Ydfors et al., 2016). Moreover, there is
indirect evidence to suggest that the protein required for ADP transport into mitochondria, adenine nucleotide translocase (ANT), is impaired with aging in housefly and rat skeletal muscle (Feng et al., 2008; Yan and Schal, 2000). Therefore, previous assessments of mitochondrial ROS emission in the absence of ADP may not adequately reflect the in vivo environment, and as a result current data from human skeletal muscle may underestimate the importance of mitochondrial ROS in the aging process.

In the present study we re-evaluated mitochondrial bioenergetics by establishing a protocol in permeabilized muscle fibers to simultaneously examine mitochondrial respiration and hydrogen peroxide (H₂O₂) emission in the presence of various substrates and ADP concentrations. Using this in vitro protocol, we assessed age-related mitochondrial defects by comparing healthy young males to healthy older males. We also examined whether potential age-related defects in mitochondrial bioenergetics could be improved over 12 weeks of resistance exercise training. We provide compelling evidence that although the capacity for mitochondrial ROS emission is not increased with aging, mitochondrial ADP sensitivity is impaired, such that mitochondrial ROS, and the fraction of electron leak to ROS, are increased in the presence of virtually all ADP concentrations examined. In addition, although resistance-type exercise training improved several aspects of muscle health in older individuals, the fraction of electron leak to ROS, mitochondrial H₂O₂ emission rates in the presence of ADP, and muscle redox stress were unaltered, suggesting an increase in mitochondrial ROS accompanies the aging process.

RESULTS

Establishment of an In Vitro Methodology to Assess Mitochondrial Bioenergetics

We first aimed to establish a methodology to simultaneously measure mitochondrial ROS and oxygen consumption rates in the presence of mixed substrates, including ADP. The provision of succinate induces reverse electron flow, preventing the ability to assess respiratory sensitivity in the absence of rotenone, which does not occur physiologically. Therefore, we aimed to use pyruvate to induce forward electron flow after the addition of succinate, and in this manner a single protocol would generate the typical capacity estimate for mitochondrial ROS (succinate supported) and a mixed substrate-supported ROS rate (succinate and pyruvate/malate). Although forward electron flow has been suggested to attenuate succinate-induced ROS (Lambert and Brand, 2004), the provision of pyruvate did not alter H₂O₂ emission rates in human skeletal muscle fibers (Figure 1A). We therefore reasoned that previous studies used higher concentrations of malate, and the resulting accumulation of oxaloacetate, inhibited SDH activity. To test this we titrated in malate, and report that at higher concentrations mitochondrial ROS is attenuated (Figure 1B), suggesting oxaloacetate, and not forward electron flow, attenuates mitochondrial ROS. Given the establishment that mitochondrial ROS is detectable with a mixed complex I (CI) and complex II (CII) protocol, we next titrated ADP after the addition of pyruvate + malate to determine the ability of ADP to stimulate respiration (Figure 1C). Using this approach, maximal ADP stimulated respiration ~4-fold and completely ablated H₂O₂ emission (Figure 1C), while submaximal ADP concentrations altered these bioenergetic responses to various degrees and enabled estimates of ADP sensitivity to stimulate respiration (apparent ADP Kₘ, Figure 1D) and attenuate mitochondrial ROS emission (apparent half maximal inhibitory concentration [IC₅₀]; Figure 1E) to be calculated.

Examination of Mitochondrial Bioenergetics with Aging

We next examined mitochondrial bioenergetics with aging using this methodology. To accomplish this we compared bioenergetic responses of permeabilized skeletal muscle fibers in healthy young (26 ± 2 years) and older (70 ± 1 years) individuals who displayed greater BMI, homeostatic model assessment of insulin resistance (HOMA-IR) (p = 0.06), and glycated hemoglobin (HbA₁c) (Table 1). We recruited young individuals who were not well trained (peak oxygen consumption [VO₂peak] 44 ± 3 mL/kg/min) in an attempt to minimize differences in physical fitness between the two age groups. Nevertheless, the skeletal muscle of older individuals displayed reductions in the proportion, and cross-sectional area, of type II fibers (Figures 1A and 1B), supporting previous reports suggesting the preferential atrophy of type II fibers with aging (Klitgaard et al., 1990; Nilwik et al., 2013).

We first examined classical assessments of mitochondrial bioenergetics in these individuals and found no differences between young and old with respect to respiration in the presence (state III) or absence (state II) of ADP or in maximal oxidative phosphorylation or respiratory control ratio (RCR) (Figure 2C). In addition, maximal succinate and succinate + pyruvate H₂O₂ emission rates (Figure 2D), as well as the abundance of oxidative phosphorylation (OXPHOS) proteins (Figures 2E and 2F), were not altered with aging. However, although the provision of pyruvate did not stimulate H₂O₂ emission in younger individuals, pyruvate stimulated H₂O₂ emission in older individuals (Figure 2D), suggesting a greater contribution from either pyruvate dehydrogenase or CI. Regardless, these data support recent reports of unaltered mitochondrial respiration and ROS emission capacities with aging (Kent-Braun and Ng, 2000; Larsen et al., 2012). However, a major control point in bioenergetics is the movement of ADP into the mitochondria, and therefore we next analyzed the dynamic response of mitochondria to submaximal ADP concentrations. Titrating ADP revealed the expected Michaelis-Menten kinetic curve (Figure 3A, inset), which was used to estimate the apparent sensitivity for ADP to stimulate respiration (apparent Kₘ). Although maximal respiration was not different (Figure 2A), the apparent ADP Kₘ was ~25% higher in older individuals (Figures 3A and 3B), indicating an attenuation in ADP sensitivity. This is supported by reduced absolute respiration, and attenuated stimulation of respiration, in the presence of ADP concentrations, indicative of resting muscle (Figure 3C). Older muscle also displayed a rightward shift in the H₂O₂ curve in the presence of ADP (Figure 3D), such that the apparent IC₅₀ value for ADP to attenuate mitochondrial ROS emission was increased ~8-fold with aging (Figure 3E). This basic pattern was also apparent when examining mitochondrial H₂O₂ emission rates in the presence of ADP concentrations that correspond to resting free ADP
concentrations in muscle (Figure 3F), as absolute emission rates were higher, while the decrease in ROS was lower, in the presence of 25 and 175 μM ADP (Figure 3F). In addition, the fraction of electron leak to H$_2$O$_2$ was unaltered with aging in the absence of ADP, but it was higher with aging in the presence of every ADP concentration examined (Figure 3G), which corresponded to a higher concentration of ADP that was required to decrease the fraction of electron leak to ROS by half (Figures 3H and 3I). Because ATP synthase content was not reduced (Figure 2E), we evaluated the expression of proteins involved in the transport of ADP into mitochondria, a process that requires ANT (inner mitochondrial membrane) and voltage-dependent anion channel (VDAC; outer mitochondrial membrane), and augmented by mitochondrial creatine kinase (mi-CK). Although ANT isoforms and mi-CK content were not different between age groups, VDAC was reduced in older muscle (Figures 3J and 3K), which may contribute to the observed attenuation in ADP sensitivity. Altogether, these data highlight that although maximal mitochondrial respiration and ROS emission rates are not altered with aging, the sensitivity to ADP is dramatically impaired, resulting in an increase in ROS emission in the presence of submaximal ADP concentrations. Given the disparity between the observed maximal and submaximal ROS rates, we evaluated markers of cellular oxidative stress. Specifically, we determined the glutathione (GSH) and glutathione disulfide (GSSG) as well as 4-hydroxynonenal (4HNE) protein adduct content within the skeletal muscle of young and old individuals. Although GSH and GSSG were increased with aging, the ratio of GSH to GSSG was reduced with aging (Figure 4A), suggesting the induction of oxidative stress in vivo. In support of this, 4HNE protein adduct content was also increased in older individuals (Figure 4B). These responses were not associated with reductions in the content of antioxidant enzymes (Figure 4C). Altogether, these data indicate oxidative stress occurs with aging, supporting the current in vitro assessments of mitochondrial ROS in the presence of ADP.

**Determining the Ability of Exercise Training to Rectify the Derangements in Mitochondrial ADP Sensitivity with Aging**

Endurance training, while inducing mitochondrial biogenesis, has been shown to reduce the intrinsic sensitivity of mitochondria to
ADP (Ludzki et al., 2015). Alternatively, resistance exercise training increases muscle mass, glucose tolerance, and functional capacity and is therefore an effective strategy to combat the aging process. We therefore used a 12-week resistance-type exercise training program in different older individuals to establish the influence on mitochondrial ADP sensitivity. Resistance training increased (p < 0.05) lean mass (+1.1 ± 0.3 kg), appendicular lean mass (+1.1 ± 0.2 kg), and leg strength (+1.8 ± 2.5 kg) and tended (p = 0.5) to increase OXPHOS protein content (Figures 5G and 5H) were all improved. However, despite the improvement in mitochondrial ADP sensitivity, (3) a greater fraction of electron leak to ROS, (4) oxidative stress. Moreover, we show that although resistance-exercise training (5) increased maximal mitochondrial respiration, (2) an increase in mitochondrial ADP sensitivity to be improved. In addition, exercise training did not alter the efficiency of the ETC, as the fraction of electron leak to ROS was not altered at any ADP concentration examined (Figures 5J and 5K).

When examining the abundance of ADP transport proteins, despite the improvement in ADP sensitivity after resistance training, VDAC and ANT protein contents were not altered (Figures 5L and 5M), suggesting a change in these proteins is not required for ADP sensitivity to be improved.

Given the disparity between maximal and submaximal mitochondrial ROS emission rates, similar to the comparison between young and old individuals, we examined markers of redox stress. Despite the strong trend for an increase in maximal succinate-supported ROS (Figure 5C), the ratio of GSH to GSSG (Figure S1A), and 4HNE protein adduct content (Figure S1B) were not altered with exercise training, while superoxide dismutase 2 (SOD2) increased (Figure S1C). Once again these markers of in vivo redox stress better align with the unaltered ROS in the presence of ADP than with the capacity to produce ROS. This relationship is also highlighted by collapsing all of the current data: whereas maximal ROS is not altered with aging (Figure 6A), ROS in the presence of ADP is consistently higher (Figure 6B), regardless of training status, which corresponds with a lower GSH/GSSG ratio (Figure 6C).

### DISCUSSION

The present data establish that although maximal mitochondrial respiration and H$_2$O$_2$ are not altered with aging, mitochondrial bioenergetics in the presence of non-saturating ADP concentrations are dramatically impaired in skeletal muscle tissue of older individuals. Specifically, we provide evidence that because of a reduction in mitochondrial ADP sensitivity, aging is associated with (1) a decrease in mitochondrial respiration, (2) an increase in mitochondrial ROS emission, (3) a greater fraction of electron leak to ROS, and subsequently (4) oxidative stress. Moreover, we show that although resistance-exercise training (5) increased the capacity for mitochondrial bioenergetics (respiration and ROS), (6) mitochondrial ADP sensitivity was improved (decreased $K_m$ and IC$_{50}$, and as a result (7) mitochondrial H$_2$O$_2$ emission and the fraction of electron leak to ROS were not altered in the presence of ADP, and therefore (8) the oxidative state of the muscle was not affected by resistance training.

### Table 1. Basic Subject Characterization

<table>
<thead>
<tr>
<th>Basic Characterization</th>
<th>Old Pre-training</th>
<th>Old Post-training</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>26 ± 2</td>
<td>70 ± 1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.6 ± 3.1</td>
<td>76.4 ± 1.6</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>22.6 ± 0.8</td>
<td>25.2 ± 0.7</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>4.7 ± 0.2</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>Blood insulin (mU/L)</td>
<td>7.4 ± 1.3</td>
<td>9.2 ± 1.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.8 ± 0.3</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>59.5 ± 1.6</td>
<td>61.0 ± 1.7</td>
</tr>
<tr>
<td>Appendicular lean mass (kg)</td>
<td>26.3 ± 0.7</td>
<td>27.4 ± 0.6</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>16.2 ± 1.1</td>
<td>15.9 ± 1.0</td>
</tr>
<tr>
<td>Maximal leg extension (kg)</td>
<td>82.5 ± 3.1</td>
<td>100.3 ± 3.4</td>
</tr>
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n = 10; values are presented as mean ± SEM.

$^a$Significant difference (p < 0.05) from young or pre-training.
Altogether, these data highlight in humans that there is an age-related increase in mitochondrial H$_2$O$_2$ emission that appears to be resistant to improvements following 12 weeks of resistance-exercise training and likely contributes to the greater oxidative stress that occurs in senescent muscle.

**Skeletal Muscle Mitochondrial H$_2$O$_2$ Emission Is Increased with Aging**

The notion that increased mitochondrial ROS emission contributes to aging in humans remains a current topic of scientific debate (Cartee et al., 2016) as in vitro assessments of mitochondrial ROS emission in humans are unaltered with aging (Capel et al., 2005; Distefano et al., 2017; Gouspillou et al., 2014). In the present study, although we report unaltered maximal mitochondrial H$_2$O$_2$ emission rates with aging (Capel et al., 2005; Distefano et al., 2017; Gouspillou et al., 2014), we provide compelling evidence that mitochondrial ROS emission is increased with aging when the in vitro environment better matches in vivo resting muscle. Specifically, by titrating ADP in the presence of mixed substrates, we were able to reveal an impairment in the ability of ADP to reduce ROS emission in older individuals. Although largely unstudied, ADP sensitivity represents a major control point in metabolic homeostasis, as the transport of ADP into mitochondria, and the subsequent binding of ADP to F1F0 ATP synthase, decreases membrane potential and the overall rate of superoxide production (Bornhövd et al., 2006), while stimulating aerobic metabolism. The present data strongly suggest that alterations in ANT or ATP synthase content do not contribute to the impairment in ADP sensitivity with aging or improvement with resistance training.

Figure 2. Basic Characterization of Muscle with Aging
(A–D) Immunohistochemical assessments of skeletal muscle fiber type and cross-sectional area (A and B), maximal respiration (JO$_2$) (C) and hydrogen peroxide (H$_2$O$_2$) emission rates (D) are not altered with aging.
(E and F) Markers of the ETC.

*Significant difference (p < 0.05) from young.

n = 10; values are reported as mean ± SEM.
Recent literature has revealed in diverse models that ADP sensitivity is highly regulated, as acute and chronic aerobic exercise training (Ludzki et al., 2015; Perry et al., 2012), reactive lipids (Ludzki et al., 2015), and diabetic rats (Smith et al., 2013) have all displayed altered mitochondrial responses to submaximal ADP. Clearly the mechanisms regulating ADP sensitivity need to be delineated given the diverse implications to metabolic homeostasis.

In addition to the observed attenuated ADP sensitivity contributing to redox stress with aging, older individuals also displayed an ability for pyruvate/malate to stimulate mitochondrial H₂O₂ emission, suggesting either a greater contribution from pyruvate dehydrogenase or an increase in superoxide production from the FMN site on CI. Numerous studies have demonstrated that pyruvate dehydrogenase content and activity either remain stable or decline throughout the lifespan (Gurd et al., 2008; Rasmussen et al., 2003), while in the present study, a marker of CI content was unaltered with aging, suggesting that the greater drive on ROS emission with pyruvate/malate in the older individuals might be caused by impaired CI function (Picard et al., 2010). In support, it has been previously suggested that an overly reduced FMN site on CI may be the prevailing source of ROS emission (Brand, 2010), particularly because CI is susceptible to cysteine modifications (Staunton et al., 2011). Further to this, one study in humans (Short et al., 2005), and various rodent models of aging (Desai et al., 1996; Kumaran et al., 2004;
Mansouri et al., 2006; Picard et al., 2010), have reported dramatic declines in CI activity later in the lifespan, while caloric restriction, which has been well defined to prolong mammalian lifespan by 30%–50%, has also been shown to reduce CI ROS emission in rodent heart tissue (Gredilla et al., 2001). The topology of ROS production with aging remains to be fully delineated, but the stimulatory effect of pyruvate with aging, combined with the attenuated response to ADP, likely contributes to the observed oxidative stress in older individuals.

**Skeletal Muscle Mitochondrial Respiration Is Decreased with Aging**

Aging was originally believed to be associated with decreased mitochondrial respiratory capacity, as various markers of mitochondrial content are reduced in older individuals. However, contemporary research has shown that maximal mitochondrial respiration is not altered with aging (Capel et al., 2005; Distefano et al., 2017; Gouspillou et al., 2014), suggesting that a reduction in respiratory capacity is not a primary cause of the aging process. In the present study, we support these findings, as there were trends for OXPHOS content to be reduced, while we did not observe age-related alterations in leak respiration or respiration in the presence of saturating ADP concentrations (maximal respiration). These data are in stark contrast to in vivo reports of mitochondrial function using $^{31}$P-magnetic resonance spectroscopy (MRS), which have consistently suggested impaired mitochondrial function with aging in resting (Fleischman et al., 2010) and contracting muscle (Choi et al., 2016). In the present study, respiration was lower in the presence of non-saturating ADP concentrations with aging, and the apparent sensitivity of mitochondria for ADP to stimulate respiration was decreased (apparent $K_m$ increased $\sim$25%). These data highlight that mitochondrial bioenergetics are impaired in the presence of non-saturating ADP in older individuals and likely contributes to the observed impairment in mitochondrial function with aging as assessed by $^{31}$P-MRS (Choi et al., 2016; Fleischman et al., 2010).

**Resistance Exercise Training Does Not Fully Recover Mitochondrial Bioenergetics with Aging**

Physical activity profoundly affects the capacity of mitochondrial ROS emission and respiration (Gouspillou et al., 2014; Gram et al., 2015; Porter et al., 2015b). Resistance exercise training has also been well established as a particularly effective strategy to increase muscle mass, strength, and metabolic function in older individuals (Leenders et al., 2013a; Porter et al., 2015b). In the present study, ten different older individuals completed 12 weeks of resistance training, which resulted in increased lean body mass and leg extension one-repetition maximal strength along with a concomitant reduction in body fat percentage. In addition, resistance training increased maximal mitochondrial respiration and ROS emission and strongly tended to increase OXPHOS content, all indicative of exercise-induced mitochondrial biogenesis. Resistance training has also been shown to increase mtDNA content in young and old individuals, while not altering respiratory function in isolated mitochondria (Robinson et al., 2017), further suggesting the increase in respiration observed in the present study using permeabilized muscle fibers reflects the induction of mitochondrial bioenergetics.
biogenesis, as opposed to an intrinsic alteration within mitochondria. Although aging was associated with a reduction in ADP sensitivity, exercise training increased the ability of submaximal ADP concentrations to stimulate respiration and attenuate ROS emission. In an attempt to explain these findings we examined the content of proteins required for ADP transport across the mitochondrial membrane. However, the expression of these proteins did not align with the observed bioenergetic alterations, as the content of ANT isoforms did not change with any condition, and although VDAC was reduced with aging, training did not alter the content of this protein despite improving ADP sensitivity. In contrast, the observed changes in ADP sensitivity with aging and following prolonged resistance training aligned with the relative proportion of type I fibers. These data raise the possibility that the observed changes in mitochondrial bioenergetics are influenced in a fiber-specific manner. Interestingly, in rats, the red portion of skeletal muscles, which have a higher proportion of type I fibers, display greater mitochondrial H$_2$O$_2$ emission potential within the matrix and lower mitochondrial ADP sensitivity (Anderson and Neufer, 2006; Zoll et al., 2003), suggesting mitochondrial ROS may impair ADP sensitivity, creating a vicious cycle. It is therefore possible that redox-induced inhibition of ANT is an explanation for the observed changes in the present study, as there is also indirect evidence to suggest that ANT is impaired through redox modifications with aging in housefly and rat skeletal muscle (Queiroga et al., 2010; Yan and Sohal, 1998), and ANT has three known cysteine sites located on the...
matrix-facing surface that can influence ADP transport (McStay et al., 2002). In further support of this, resistance training increased the content of SOD2, a matrix localized antioxidant in association with reducing the apparent increased the content of SOD2, a matrix localized antioxidant 

et al., 2002 ). In further support of this, resistance training also increased the capacity for mitochondrial H2O2, the improvement in ADP sensitivity did not attenuate H2O2 emission in the presence of sub-saturating ADP concentrations. These data suggest that the in vivo oxidative state of the muscle was not affected by training, an interpretation supported by the observed unaltered GSH/GSSG ratios and 4HNE protein adduct content following training. In addition, exercise training did not improve the apparent ETC efficiency, as the fraction of electron leak to H2O2 was unchanged across a range of biologically relevant ADP concentrations. Therefore, although resistance training improved muscle strength and mitochondrial ADP sensitivity in older individuals, H2O2 emission in the presence of sub-saturating ADP concentrations was not altered, and therefore the oxidative state of the skeletal muscle was not improved. These data imply that the rate of H2O2 emission from mitochondria is consistently higher in older individuals, regardless of resistance training status. However, future work should elucidate the ability of aerobic and high-intensity interval training to improve ADP sensitivity and redox balance in aged muscle, as these forms of training regimes have been shown to rectify many age-associated mitochondrial abnormalities (Gram et al., 2015; Robinson et al., 2017), and high-intensity interval training has been shown to dramatically improve mitochondrial ADP sensitivity in young healthy individuals (Ydfors et al., 2016).

Conclusions

Altogether, the assessment of mitochondrial bioenergetics in the presence of sub-saturating ADP concentrations has revealed that there are age-associated impairments in mitochondrial bioenergetics, which are not fully recovered with prolonged resistance-type exercise training. The mechanism for the attenuation in ADP sensitivity remains unknown, but oxidative damage has been proposed as a likely explanation (Feng et al., 2008; Yan and Sohal, 2000). Regardless of this knowledge gap, the present data imply that an increase in mitochondrial ROS is associated with the primary aging process. Moreover, despite the inability of resistance training to rectify age-related mitochondrial ROS emission, older individuals experienced favorable changes in muscle mass, strength, and fat mass, reinforcing the importance of a physically active lifestyle throughout the lifespan.

EXPERIMENTAL PROCEDURES

Subjects

Ten healthy, young men (26 ± 2 years, 71.6 ± 3.1 kg, 22.6 ± 0.8 kg/m2) and ten healthy older men (70 ± 1 years, 76.4 ± 1.6 kg, 25.2 ± 0.7 kg/m2) were included in the cross-sectional, aging comparison of this study. Ten older men (70 ± 1 years, 78.8 ± 2.5 kg, 25.8 ± 0.6 kg/m2) participated in the 12-week progressive resistance exercise training program. Subject characteristics of the study participants are presented in Table 1. All muscle biopsies were performed in an overnight fasted and rested state between 8 a.m. and 11 a.m. Experimental procedures were approved by the Medical Ethical Committee of the Maas- 

tricht University Medical Centre, the Netherlands, and conformed to standards for the use of human subjects in research as outlined in the most recent version of the Helsinki Declaration.

Pretesting and Study Design

All participants were screened for medical issues and excluded if any chronic diseases were present before inclusion. Anthropometric measurements, maximal strength assessment, dual-energy x-ray absorptiometry (DEXA) scans, and muscle biopsy and blood samples were collected as previously reported (Leenders et al., 2013a; Snijders et al., 2015). Supervised resistance-type exercise training was performed three times per week for a 12-week period, consisting of three or four sets (eight to ten repetitions per set) of leg press and leg extension, in addition to upper body exercises. The workload was progressively increased throughout the program to maintain 80% one-repetition maximum (1RM). All training sessions were performed in the morning between 8:30 a.m. and 11:30 a.m. On average, subjects attended 96% ± 4% of the scheduled exercise sessions.

Mitochondrial Bioenergetics

Skeletal muscle was separated under a microscope into bundles using fine- tipped forceps, treated with 30 μg/mL saponin for 30 min at 4°C, and then washed for 15 min in buffer Z (105 mM K-Mes, 30 mM KCl, 1 mM EGTA, 10 mM K2HPO4, 5 mM MgCl2, H2O, 0.005 mM pyruvate, 0.002 mM malate with 5 mg/mL fatty acid-free BSA [pH 7.4]), as previously described (Herbst et al., 2014). We have previously used exogenous hexokinase to create an “ADP clamp” within our system and reported the absence of an effect on mitochondrial ADP sensitivity (Perry et al., 2012; depicted in Figure S2). We therefore do not believe that alterations within “non-mitochondrial” sources of ADP utilization affect our current interpretations. Therefore, O2 consumption
and H₂O₂ emission were determined in buffer Z supplemented with 20 μM Amplex Red (Invitrogen, Carlsbad, CA, USA), 5 U/mL horseradish peroxidase, 40 U/mL superoxide dismutase, and 25 μM blebbistatin, using an Oxigraph-2 K with a fluorometry modular attachment (Oroboros Instruments, Innsbruck, Austria). Experiments were conducted at 37 °C and commenced with mild hyper-oxygenation to prevent re-oxygenation during an experiment (250 μM O₂) in the presence of various substrates, including 10 μM cytochrome c to ensure outer mitochondrial membrane integrity. The rate of H₂O₂ emission was calculated from a standard curve established with the same reaction conditions using the DatLab software (Oroboros Instruments) after subtracting the fiber background. All fibers were weighed in buffer Z before the start of an experiment to normalize respiration to muscle bundle weight.

Immunohistochemistry
From the pre- and post-training muscle biopsy samples, 5-mm-thick cryosections were cut at 220 °C. Samples collected from each subject before and after the 12-week training intervention were mounted on uncoated glass slides. Muscle biopsy specimens were stained for DAPI, myosin heavy chain 1 and analyzed and interpreted the data. G.P.H., A.M.H., P.M.M., M.L.D., L.B.V., and L.J.C.v.L. performed experiments and analyzed and interpreted the data. G.P.H. designed the study and wrote the manuscript. A.M.H. contributed to writing the manuscript. G.P.H., A.M.H., P.M.M., M.L.D., L.B.V., and L.J.C.v.L. edited and approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: March 16, 2017
Revised: December 13, 2017
Accepted: February 16, 2018
Published: March 13, 2018

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