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Impaired mitochondrial degradation by autophagy in the skeletal muscle of the aged female interleukin 10 null mouse

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ABSTRACT

Mitochondrial dysfunction, chronic inflammation and muscle aging are closely linked. Mitochondrial clearance is a process to dampen inflammation and is a critical pre-requisite to mitobiogenesis. The combined effect of aging and chronic inflammation on mitochondrial degradation by autophagy is understudied. In interleukin 10 null mouse (IL-$10^{tm/tm}$), a rodent model of chronic inflammation, we studied the effects of aging and inflammation on mitochondrial clearance. We show that aging in IL-$10^{tm/tm}$ is associated with reduced skeletal muscle mitochondrial death signaling and altered formation of autophagosomes, compared to age-matched C57BL/6 controls. Moreover, skeletal muscles of old IL-$10^{tm/tm}$ mice have the highest levels of damaged mitochondria with disrupted mitochondrial ultrastructure and autophagosomes compared to all other groups. These observations highlight the interface between chronic inflammation and aging on altered mitochondrial biology in skeletal muscles.

1. Introduction

The age-related inflammatory myopathies are strongly associated with mobility impairment, falls, and frailty (Fried et al., 2001; Walston et al., 2002; Franceschi and Campisi, 2014; Cohen et al., 2003; Cruz-Jentoft et al., 2010; Bandeen-Roche et al., 2006). The mitochondria play a central role in the muscle inflammatory aging process and serve as a link between aging and inflammation (Peterson et al., 2012). Evidence suggests that changes in mitochondria are influenced by chronic inflammation, and as a result, the increased free radical production from dysfunctional mitochondria further activates inflammatory cascades thus creating a vicious cycle (van der Burgh et al., 2014). The clearance of damaged mitochondria is therefore a critical step in breaking this cycle and is an important pre-requisite for the generation of new mitochondria (Palikaras and Tavernarakis, 2014). The impact of chronic inflammation on mitochondrial degradation and clearance by autophagy (mitophagy) is understudied in the context of aging and frailty.

Several factors mediate the interaction between inflammation and mitophagy. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine expressed in muscle fiber membranes (Reimann et al., 2010). MIF has been shown to inhibit mitochondria-dependent death pathways (Baumann et al., 2003) and prevents apoptosis. In contrast, Nix/Bnip3L (NIP3-like protein X, NIX) (Zhang and Ney, 2008) is a mitochondrial death protein in that it triggers mitophagy and triggers apoptosis (Yussman et al., 2002). NIX and MIF have been shown to functionally and physically antagonize each other (Damico et al., 2008). The combined effects of aging and chronic inflammation on NIX/MIF and subsequently on autophagy and mitochondrial clearance in skeletal muscles are not known. Here we sought to investigate the progression and development of age-related myopathies in the pre-existing context of inflammatory conditions. To model inflammation and to study the biology linking chronic inflammation, aging, and late-life myopathy, we utilized the B6.129P2-Il10tm1Cgn/J (IL-$10^{tm/tm}$) mouse that is deficient for interleukin-10 (IL-10). The IL-$10^{tm/tm}$ mouse has a propensity to develop age-related elevated serum inflammatory cytokines (e.g. interleukin 6 (IL-6), tumor necrosis factor a (TNF-a)), muscle weakness, and higher mortality compared to C57BL/6 (B6) controls (Walston et al., 2008; Ko et al., 2012). Our prior findings including abnormal mitochondrial energy production, ATP kinetics (Akki et al., 2013) and differential expression of apoptosis and mitochondrial function genes (Walston et al., 2008) in the skeletal muscles of old IL-$10^{tm/tm}$ mice suggest that mitochondria alterations may play an important role in linking chronic inflammation and myopathy. Given this evidence, we hypothesized that in the skeletal muscles of IL-$10^{tm/tm}$ mouse, disturbance in mitochondrial ATP kinetics

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is precipitated by inflammation and age-related changes in mitochondrial clearance. In order to test these hypotheses, we sought to identify in the quadriceps femoris muscles of IL-10$^{tm/tm}$ and B6 mice, inflammation- and age-associated differences in mitochondrial degradation biology using markers of mitophagy induction (NIX), microtubule-associated protein light chain 3 autophagosomes marker (LC3), apoptosis inhibition (MIF) (Damico et al., 2008; Ding et al., 2010; Chuang et al., 2012), and changes in mitochondria ultrastructure using electron microscopy.

2. Methods

2.1. Animals

Female IL-10$^{tm/tm}$ and B6 mice (Jackson Laboratory, Bar Harbor, ME; National Institute on Aging, Bethesda, MD) were housed in specific pathogen free barrier conditions until the appropriate age was reached and then sacrificed. A cross-sectional study design was utilized to compare differences in quadriceps femoris muscle (QF) gene expression and mitochondria ultrastructure between groups (N = 3–6) of young (3–5 months-old) and old (22–24 months-old) mice. All protocols were approved by the Animal Care and Use Committee of Johns Hopkins School of Medicine.

2.2. Protein extraction/Western blot analysis

Proteins were extracted from flash frozen QF muscles using T-PER (Thermo Scientific) with the addition of protease (Complete Mini, Roche) and phosphatase (PhosStop, Roche) inhibitors. Equal concentrations of proteins were electrophoresed using Bis–Tris gels (Invitrogen), transferred onto nitrocellulose membrane, and then incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: NIX (Invitrogen: 1:5000 mouse primary, 1:10,000 goat secondary), LC3 (Cell Signaling: 1:1000 mouse primary, 1:10,000 goat secondary), MIF (Santa Cruz: 1:500 rabbit primary, 1:5000 goat secondary), and actin (Sigma: 1:10,000 rabbit primary, 1:20,000 goat secondary). HRP-conjugated secondary antibodies were used to detect bands (Amersham). Quantitative Western blot analyses were performed using ImageJ (National Institutes of Health).

2.3. Statistical analysis

One-way ANOVA with Tukey's post-hoc test or Kruskal–Wallis nonparametric analysis with a Dunnett's post-hoc test was used to determine differences among groups. When 2 groups were compared, an unpaired, 2-tailed Student's *t*-test or a Wilcoxon rank-sum test was used.

2.4. Transmission electron microscopy (TEM)

TEM was used to compare differences in mitochondrial ultrastructure and autophagosome (AP) accumulation. Data acquisition and analyses for TEM utilized multiple thin QF sections (70–90 nm) with high tissue integrity captured on representative photomicrographs (45 μm^2 of muscle tissue/image) from 10 randomly selected fields (Frazier et al., 2011). Mitochondria were identified as normal if intact, or abnormal if they had disrupted membranes, cristae depletion, and matrix dissolution (Arismendi-Morillo, 2009; Divi et al., 2007). Group differences in the frequency of mitochondria with abnormal morphology were tested by *chi-square*. AP were quantified for each mouse by averaging the number of AP detected at 10,000× magnification per 32,000 μm^2 of tissue from three distinct grids. Group differences in AP/32,000 μm^2 were tested by repeated-measure ANOVA.

3. Results

MIF is detected at higher levels in inflammatory myopathies and may function as a NIX antagonist to delay apoptosis by inhibiting mitochondria-dependent death pathway. In the IL-10$^{tm/tm}$ mouse, inflammation was associated with higher levels of skeletal muscle MIF proteins (young B6, 0.6 ± 0.1 AU vs. young IL-10$^{tm/tm}$, 1.4 ± 0.2 AU, $p < 0.05$; and old B6, 0.2 ± 0.1 AU vs. old IL-10$^{tm/tm}$, 1.1 ± 0.2 AU, $p < 0.01$; Fig. 1A). Given the antagonistic crosstalk between MIF and NIX on mitochondrial homeostasis, we quantified changes in NIX protein levels in skeletal muscles of our mouse cohorts. In contrast to MIF, the expression of NIX was the highest in the skeletal muscles of the old control mice (young B6, 0.03 ± 0.01 AU vs. old B6, 1.7 ± 0.3 AU, $p < 0.0001$; Fig. 1B). Interestingly, in old IL-10$^{tm/tm}$ mice that exhibited combined effects of aging and inflammation, the expression of NIX was lower compared to the old control mice (old IL-10$^{tm/tm}$, 0.3 ± 0.08 AU vs. old B6, 1.7 ± 0.3 AU, $P < 0.001$; Fig. 1B).

In order to determine if NIX and MIF expression in the different animal groups resulted in changes in assembly of autophagosomes (AP), we quantified changes in LC3 expression. Parallel to NIX levels, the highest expression of LC3 was seen in the old control group as compared to all the other groups (old B6, 1.9 ± 0.4 AU vs. old IL-10$^{tm/tm}$, 0.8 ± 0.3 AU, $p < 0.05$; Fig. 1C). Taken together, these data suggest that inflammation- and age-associated differences in NIX, MIF and LC3 expression are particularly pronounced in old IL-10$^{tm/tm}$ mice and that these changes may influence mitochondrial autophagy.

Parallel to MIF, NIX, and LC3 gene expression changes, more intracellular double-membrane vacuolated structures consistent with AP (Fig. 2A, B) were identified in old IL-10$^{tm/tm}$ compared to old B6 (12.7 ± 4.8 AP/32,000 μm^2 vs. 8.3 ± 3.2 AP/32,000 μm^2, $p = 0.03$; Table 1) on TEM. AP frequently localized to clusters of mitochondria (89.5% in old IL-10$^{tm/tm}$, 88.1% in old B6; Fig. 2C). Some AP appeared to contain predominately lipids (Fig. 2A, B), while most others appeared to contain mixed cellular contents and electron-dense lipofuscin-like granules (Fig. 2C) (Sandri, 2010; Masiero and Sandri, 2010). Old IL-10$^{tm/tm}$ had more AP with granular inclusions compared to old B6 (35.6% vs. 23.2%, $p < 0.015$ by *chi-square*). Normal (Fig. 2D) and abnormal, likely depolarized (Fig. 2E, F) mitochondria were present in all mice groups but the frequency of mitochondria with abnormal ultrastructure was higher in old IL-10$^{tm/tm}$ (7.55% in young IL-10$^{tm/tm}$ vs. 14.21% in old IL-10$^{tm/tm}$, $p < 0.001$) and old B6 (4.88% in young B6 vs. 7.63% in old B6, $p = 0.012$; Table 1). IL-10$^{tm/tm}$ had significantly more abnormal appearing mitochondria compared to B6 of both age groups (Table 1). Taken together, these data suggest inflammation- and age-associated differences in mitochondrial damage and clearance and mitochondrial autophagy in skeletal muscles of mice.

4. Discussion

This study suggests that in the skeletal muscles of IL-10$^{tm/tm}$ and B6 mice, there are inflammation- and age-associated changes in mitochondrial biology including mitochondrial ultrastructure (abnormal appearing mitochondria), mitophagy induction (NIX, AP and LC3), and mitochondria-dependent death pathway inhibition (MIF). In old IL-10$^{tm/tm}$ mice compared to age-matched B6 controls, these mitochondria-related changes are particularly enhanced, suggesting that inflammation and aging have an additive role in altered mitochondrial biology in skeletal muscles.

The pathogenesis of sarcopenia likely involves a number of intramuscular specific processes including oxidative stress, mitochondrial dysfunction, impaired mitochondrial turnover, and mitochondrion-mediated apoptosis (Marzetti et al., 2013a). In this study, we focused on the process of mitophagy (autophagy-mediated mitochondrial turnover) in skeletal muscles. Many studies have demonstrated aging-associated progressive accumulation of damaged macromolecule and