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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.

Keywords

IL-10; Autophagy; Mitochondria; MIF; NIX

INTRODUCTION

The age-related inflammatory myopathies are strongly associated with mobility impairment, falls, and frailty (1–6). The mitochondria plays a central role in the muscle inflammatory aging process and serves as a link between aging and inflammation (7). 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

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inflammatory cascades thus creating a vicious cycle (8). 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 (9). 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 (10). MIF has been shown to inhibit mitochondria-dependent death pathways (11) and prevent apoptosis. In contrast, Nix/Bnip3L (NIP3-like protein X, NIX) (12) is a mitochondrial death protein in that it triggers mitophagy and triggers apoptosis (13). NIX and MIF have been shown to functionally and physically antagonize each other (14). 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-Il10^{tm1Cgn/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 α (TNF- α)), muscle weakness, and higher mortality compared to C57BL/6 (B6) controls (15;16). Our prior findings including abnormal mitochondrial energy production, ATP kinetics (17) and differential expression of apoptosis and mitochondrial function genes (15) 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 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) (14;18;19), and changes in mitochondria ultrastructure using electron microscopy.

METHODS

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.

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: 1:5,000 mouse primary, 1:10,000 goat secondary), LC3 (Cell Signaling: 1:1,000 mouse primary, 1:10,000 goat secondary), MIF (Santa Cruz: 1:500 rabbit primary, 1:5,000 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).

Statistical analysis

One-way ANOVA with Tukey's post-hoc test or Kruskal-Wallis nonparametric analysis with a Dunnett's post-hoc test were 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.

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 (20). Mitochondria were identified as normal if intact, or abnormal if they had disrupted membranes, cristae depletion, and matrix dissolution (21;22). 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 $\times$ 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.

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 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, 2B) 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, 2B), while most others appeared to contain mixed cellular contents and electron-dense lipofuscin-like granules (Fig 2C) (25, 26). 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, 2F) 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.

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 (23). 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 organelle and alteration in mitophagy (24–30). Consistent with the literature, our TEM observations support the notion that aging itself is associated with progressive accumulation of damaged mitochondria and alteration in the formation of lipofuscin-laden AP in skeletal muscles. Interestingly, the IL-10^{tm/tm} mice show evidence of enhanced accumulation of damaged mitochondria and AP compared to B6 controls. A possible explanation of this IL-10 genotype effect is that chronic inflammation (i.e. elevated IL-1 β , TNF- α) inherent in IL-10^{tm/tm} mice may further impair skeletal muscle's capacity to dispose of damaged

mitochondria (15;16;31). Additionally, because IL-10 could inhibit autophagy in murine macrophages, the absence of IL-10 in IL-10^{tm/tm} mice may be associated with a disinhibition of normal mitophagy (32). Thus, the associated increase in damaged mitochondria and lipofuscin-laden AP in skeletal muscles may underscore a failure of mitochondria and AP clearance or a compensatory response to maintain homeostasis in the setting of sarcopenia-inducing processes such oxidative stress and mitochondrial dysfunction known to be present in IL-10^{tm/tm} mice. (17;27)

Although the accumulation of damaged mitochondria and increase in AP are present in both old IL-10^{tm/tm} and B6 mice, expressions of markers of mitochondrial degradation biology (i.e. NIX, LC3) are different. In old B6, age-associated elevated NIX and LC3 suggest mitophagy induction in skeletal muscles. This observation, while consistent with studies that show an increase in autophagy markers in aged muscles (33;34), contrasts with many studies that show aging-associated decline in mitophagy (24–30). A possible explanation for this discrepancy may be the extreme ages (3–5 months-old vs. 22–24 months-old) of mice used in this study. Similar increase in LC3 protein expression has been observed in the biceps femoris muscle of 22 months-old B6 compared to 3 months-old controls (33). [In contrast to B6, age-associated changes in NIX and LC3 expression are absent in skeletal muscles of old IL-10^{tm/tm} mice. These differences suggest that in IL-10^{tm/tm} mice a chronic inflammatory state may influence age-associated changes in the molecular regulation of mitochondrial degradation and turnover. While the mechanisms underlying these differences are unknown and beyond the scope of this brief report, future mechanistic experiments focused on the dynamic process of mitophagy flux are needed to help address this knowledge gap.

These findings, while certainly not conclusive, support the hypothesis that altered mitophagy may be operant in the accelerated decline in age-associated muscle weakness, abnormalities in energy generation, and frailty previously reported in the IL-10^{tm/tm} mice (15–17). While many previous studies have shown that a combination of mitochondrial and autophagy dysfunction could contribute to age-associated degenerative and neuromuscular diseases (24–26;35–38), others have shown that reduced NIX signaling may be protective (39). In the skeletal muscles of old IL-10^{tm/tm} mouse, the reduction in NIX is accompanied by TEM evidence of mitochondrial damage and altered mitophagy, and inhibition of apoptosis and mitochondria-dependent death pathway (increased anti-NIX cytokine MIF). These concurrent changes may suggest a protective role of NIX-MIF in the context of altered mitochondrial degradation and clearance in the skeletal muscles of chronically inflamed mice. In view of the mitochondrial-lysosomal axis theory of aging, whereby abnormal lipofuscin-laden autophagosomes fail to clear abnormal mitochondria, which in turn further increase oxidative stress damage and perhaps trigger apoptosis, these observations may underscore a compensatory response of skeletal muscles to maintain cellular homeostasis in the setting of chronic inflammation and oxidative stress. (27).

Aging, chronic inflammation and mitochondrial dysfunction are closely linked. Given that the IL-10^{tm/tm} mouse demonstrates key features of frailty including early onset muscle weakness (15), chronic inflammation (15;16), and ATP kinetics impairment, future studies utilizing this mouse as an *in vivo* model to explore molecular mechanisms connecting the

dynamic process of mitophagy flux, mitochondrial energy production, chronic inflammation, loss of muscle mass, and frailty are merited.

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Fig 1. Aged and inflamed skeletal muscles show changes in MIF, NIX, and LC3 expression
 Western blot analyses of skeletal muscle protein extracts using antibodies against (A) MIF, (B) NIX, and (C) LC3. Actin was used as a loading control. Relative expression was calculated in arbitrary units (AU). (D) A representation of the MIF, NIX, and LC3 Western blots. Data are means $\pm$ SEM (n=3–5 animals) *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Fig 2.

Altered mitochondrial ultrastructure and autophagosomes with aging and/or inflammation. TEM assessment of autophagosomes (AP) and mitochondrial morphology in quadriceps femoris muscles of female IL-10^{tm/tm} and B6 mice. (A) Photomicrograph demonstrating the presence of an intracellular, vacuolated, double-membrane AP (black arrow, 30,000 $\times$) in a longitudinal skeletal muscle section of a 23 months-old IL-10^{tm/tm} mouse. (B) Double-membrane structure (white arrow, 200,000 $\times$) surrounding an AP. (C) AP with electron-dense granular inclusions (white arrow heads, 25,000 $\times$) located in proximity of clusters of mitochondria (black arrow heads). (D) Mitochondria with normal ultrastructure including intact mitochondrial membranes, cristae, and matrix (black arrow, 80,000 $\times$) in the skeletal muscle of a 23 months-old B6 mouse. (E, F) Mitochondria with abnormal structure including disrupted membrane (white arrows), loss of cristae, and matrix dissolution (asterisks) in 23 months-old B6 (E, 100,000 $\times$) and IL-10^{tm/tm} (F, 80,000 $\times$) mice.

Autophagosome (AP) quantification and mitochondria morphology in quadriceps femoris muscles demonstrating an age and genotype effect.

Table 1

Genotype and Age	Mitochondria Morphology			Comparison p Value		
	Normal	Abnormal	Percent Abnormal	IL-10 ^{tm/tm} 3 months-old	IL-10 ^{tm/tm} 23 months-old	B6 23 months-old
IL-10 ^{tm/tm} 3 months-old	784	64	7.55	–		
IL-10 ^{tm/tm} 23 months-old	1,098	182	14.21	<0.001*	–	
B6 3 months-old	799	41	4.88	0.03*	<0.001*	–
B6 23 months-old	1,476	122	7.63	–	<0.001*	0.012*
Autophagosomes per 32,000 μm^2						
IL-10 ^{tm/tm} 3 months-old	0			–		
IL-10 ^{tm/tm} 23 months-old	12.7 $\pm$ 4.8			<0.001*	–	
B6 3 months-old	0			–	<0.001*	–
B6 23 months-old	8.3 $\pm$ 3.2			<0.001*	0.03*	<0.001*

Asterisks indicate statistically significant p values. Sample size was 4 for 3 months-old B6 and IL-10^{tm/tm} and 6 for 23 months-old B6 and IL-10^{tm/tm} mice.