Adaptive plasticity of autophagic proteins to denervation in aging skeletal muscle

Michael F. O'Leary, Anna Vainshtein, Sobia Iqbal, Olga Ostojic, and David A. Hood

School of Kinesiology and Health Science, and Muscle Health Research Centre, York University, Toronto, Ontario, Canada

Submitted 16 July 2012; accepted in final form 30 November 2012

O’Leary MF, Vainshtein A, Iqbal S, Ostojic O, Hood DA. Adaptive plasticity of autophagic proteins to denervation in aging skeletal muscle. Am J Physiol Cell Physiol 304: C422–C430, 2013. First published December 5, 2012; doi:10.1152/ajpcell.00240.2012.—Aging muscle exhibits a progressive decline in mass and strength, known as sarcopenia, and a decrease in the adaptive response to contractile activity. The molecular mechanisms mediating this reduced plasticity have yet to be elucidated. The purposes of this study were 1) to determine whether denervation-induced muscle disuse would increase the expression of autophagy genes and 2) to examine whether selective autophagy pathways (mitophagy) are altered in aged animals. Denervation reduced muscle mass in young and aged animals by 24 and 16%, respectively. Moreover, young animals showed a 50% decrease in mitochondrial content following denervation, an adaptation that was not matched by aged animals. Basal autophagy protein expression was higher in aged animals, whereas young animals exhibited a greater induction of autophagy proteins following denervation. Localization of LC3II, Parkin, and p62 was significantly increased in the mitochondrial fraction of young and aged animals following denervation. Moreover, the unfolded protein response marker CHOP and the mitochondrial dynamics protein Fis1 were increased by 17- and 2.5-fold, respectively, in aged animals. Lipofuscin granules within lysosomes were evident with aging and denervation. Thus reductions in the adaptive plasticity of aged muscle are associated with decreases in disuse-induced autophagy. These data indicate that the expression of autophagy proteins and their localization to mitochondria are not decreased in aged muscle; however, the induction of autophagy in response to disuse, along with downstream events such as lysosome function, is impaired. This may contribute to an accumulation of dysfunctional mitochondria in aged muscle. reactive oxygen species; muscle atrophy; mitochondria; mitophagy; apoptosis

SKELETAL MUSCLE IS A REMARKABLY plastic tissue that undergoes a striking transformation in response to decreases in contractile activity. This distinctive response is attributable to the multinucleated composition of muscle fibers and the coordinated activation of several catabolic signaling pathways. Although muscle retains its adaptability throughout the life of an organism, tissue malleability is reduced with advancing age (4, 22). Aged muscle is further affected by an age-associated loss of skeletal muscle mass and strength, a condition known as sarcopenia (8, 22, 42). Although the precise cellular mechanisms responsible for mediating sarcopenia have yet to be fully elucidated, several studies have implicated decreases in mitochondrial function and a corresponding increase in mitochondrial mediated cell death (apoptosis) as factors contributing to this age-induced decline (4, 8). Indeed, mitochondrially mediated apoptosis can be activated by increases in reactive oxygen species (ROS), which have also been associated with several other deleterious effects, including the oxidation of mitochondrial DNA, lipids, and proteins (10, 39). Our laboratory has shown that mitochondria from aged muscle generate more ROS, possess a lower mitochondrial membrane potential (ΔΨ), and release greater amounts of proapoptotic proteins than their younger counterparts (4). This age-induced shift in apoptotic sensitivity is further supported by investigations showing that aged muscle exhibits greater caspase-3 activity (30) and DNA fragmentation (7). Thus the age-associated reductions in skeletal muscle mass are likely attributable, in part, to increases in mitochondrially mediated apoptosis.

Similar to the effects of aging, chronic muscle disuse is a potent stimulus that results in a decrease in the expression of nuclear genes encoding mitochondrial proteins (34). This contributes to an increase in mitochondrial ROS production and increases mitochondrially mediated apoptosis (1). Moreover, muscle disuse and aging also have similar negative effects on mitochondrial content (4, 41) that have been partially attributed to decreases in peroxisome proliferator-activated receptor-γ coactivator-1α (2, 4), an important regulator of the expression of nuclear genes encoding mitochondrial proteins, and an increase in mitochondrial protein degradation (16, 31). However, the exact mechanisms mediating mitochondrial degradation in response to disuse and aging are unknown. Recent investigations have implicated autophagy as an essential quality-control manager of mitochondrial content (12). General autophagy is known as a protein and organelle degradation pathway characterized by its encapsulation of identified cytoplasmic material in double-membrane vesicles, which are subsequently delivered to lysosomes for digestion (12). With respect to mitochondrial turnover, a selective form of autophagy known as mitophagy (20) acts to remove only dysfunctional mitochondria in response to elevations in ROS (28, 32) and a loss of ΔΨ. The dissipation of ΔΨ has been cited as an important molecular signal that functions to recruit Parkin, an E3 ubiquitin ligase, to the mitochondrial membrane (27). Ubiquitinated proteins are recognized by p62 and LC3II, the latter being a protein necessary for construction of the autophagosomal membrane that encases mitochondria. In addition to these markers of mitophagy, several other proteins have an important role in mediating vesicle nucleation (Beclin1 and ULK1) and LC3 maturation [autophagy-related 7 (ATG7)] (43). While many of these factors associated with mitophagy have been studied in a variety of cell types, there has yet to be a thorough investigation of the effects of denervation and age on the regulation of mitochondrial degradation in skeletal muscle.

Although ROS have a defined role in mitophagy (23), several additional intracellular processes contribute to mitochondrial degradation. For example, the molecular interplay...
between mitochondrial fission and fusion has been shown to orchestrate the segregation and removal of damaged mitochondria in concert with mitophagy (37). Specifically, the cleavage of mitochondria, through fission, acts as a mechanism to isolate mitochondria with a decreased ΔΨ and prevents these mitochondrial fragments from rejoining the organelle reticulum (37). In conjunction with these events, aging has been shown to be associated with an impaired unfolded protein response (UPR) (26), which acts as a quality-control mechanism for protein synthesis and retains the ability to trigger autophagy (44). However, the exact influence of mitochondrial dynamics and the UPR in regulation of mitochondrial degradation in aged muscle and in response to disuse has not been investigated.

The dramatic effects of aging on the physiological and biochemical aspects of muscle have an important role in regulating the progression of sarcopenia and in restricting the plasticity of aged muscle (22). Therefore, the purposes of this study were to assess the adaptive potential of young (5 mo) and aged (35 mo) muscle to 7 days of denervation and to ascertain whether regulators of autophagy are altered as a consequence of aging. We hypothesized that aged muscle would undergo less atrophy and exhibit less autophagy induction in response to the imposed stress of denervation-induced muscle disuse than young muscle. Our results provide insight into the expression of autophagy proteins in muscle and extend our previous conclusions on the range of adaptive plasticity of aged skeletal muscle.

MATERIALS AND METHODS

Animals and surgery. Male Fischer 344 Brown Norway rats were obtained from the National Institute on Aging (Bethesda, MD) at 5 mo of age (young, n = 10) and 35 mo of age (aged, n = 10), housed individually, and given food and water ad libitum. Denervation surgery, conducted as previously described (1), was approved by the York University Animal Care Committee in accordance with Canadian Council of Animal Care guidelines. Briefly, animals were maintained under anesthetic using isoflurane gas. Under sterile conditions, a 1- to 2-cm incision was made, and a 0.25-cm portion of the common peroneal nerve was excised to effectively denervate the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. The muscle incision was closed using sutures, and the skin was closed using staples. After 7 days of denervation-induced muscle disuse, the TA muscles were extracted, weighed, and used for mitochondrial isolation, while the EDL muscles were immediately sectioned for electron microscopy or immediately frozen for protein and biochemical analyses.

Mitochondrial isolation. After muscle excision, TA muscles were blotted dry, weighed, minced, and subjected to differential centrifugation to separate the tissue into mitochondrial and cytosolic fractions, as previously described (38). Briefly, after the TA muscles were minced, they were homogenized using a Teflon pestle and mortar and supplemented with a cocktail of protease inhibitors (1 mM PMSF, 10 μM leupeptin, 1.5 μM aprotinin, 1.5 μM pepstatin A, and 1 mM sodium orthovanadate). The homogenate was centrifuged at 1,000 g for 10 min at 4°C to pellet the nuclei. The supernate was carefully removed and centrifuged at 16,000 g for 20 min at 4°C to pellet the mitochondrial fraction. The supernate was removed and spun again at 16,000 g for 20 min at 4°C to pellet any residual mitochondrial mitochondria. The resulting supernate was considered to be an enriched cytosolic fraction. The mitochondrial pellet was resuspended twice in a fourfold dilution of MIB and centrifuged at 16,000 g for 20 min at 4°C after each resuspension. The supernate was finally removed, and the mitochondrial pellet was resuspended in MIB. Mitochondria were subsequently sonicated to yield the enriched mitochondrial fraction. Protein concentrations within the samples were determined using the Bradford method. The fractions were frozen at −20°C until further use.

Enzyme activities. Cytochrome c oxidase (COX) activity was measured as previously described (41) by determining the maximal rate of oxidation of fully reduced cytochrome c at 550 nm in a multidetection microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT).

Immunoblotting. Whole EDL muscle homogenates and isolated mitochondrial protein extracts were separated using SDS-PAGE, and proteins were subsequently electroblotted onto nitrocellulose membranes. After transfer, the membranes were blocked (1 h) with a solution of 5% skim milk in 1× TBST (Tris-buffered saline-Tween 20: 25 mM Tris-HCl, pH 7.5, 1 mM NaCl, and 0.1% Tween 20). Blots were then incubated in blocking solution with antibody directed against LC3II, Beclin1, ULK1, fission 1 (Fis1), dynamin-related protein 1 (Drp1), and optic atrophy 1 (Opa1, each at 1:500 dilution), p62 and acilcin (each at 1:200 dilution), ATG7 (1:3,000 dilution), Parkin, CHOP, and binding Ig protein (BIP, each at 1:1,000 dilution), mitofusin 2 (Mfn2, 1:250 dilution), and GAPDH (1:30,000 dilution) overnight at 4°C. After three 5-min washes with TBST, blots were incubated at room temperature (1 h) with the appropriate secondary antibody coupled to horseradish peroxidase and washed again three times for 5 min each with TBST. Antibody-bound protein was revealed using the enhanced chemiluminescence method. Films were scanned and analyzed using SigmaScanPro 4 (Jandel Scientific, San Rafael, CA).

Total RNA isolation. Total RNA was isolated using TRIzol reagent (catalog no. 15590-026, Invitrogen) as previously described (19) and resuspended in 25 μl of sterile water. Total RNA concentration and purity were determined by ultraviolet photometry at 260 and 280 nm, respectively. RNA quality was verified by separation of 28S and 18S rRNA on denaturing formaldehyde-1% agarose gels.

RT-PCR. Total RNA (2 μg) was reverse-transcribed to cDNA using SuperScript III reverse transcriptase as recommended by the manufacturer (Invitrogen). For real-time PCR, mRNA expression was measured with SYBR Green chemistry. Each well contained SYBR Green SuperMix, forward and reverse primers (20 nM) designed for the genes of interest (p62 and xbp1), sterile water, and 10 ng of cDNA. The detection of all real-time PCR amplification took place in a 96-well plate using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). All samples were run in duplicate. Results are reported as fold changes using the ΔΔCt method, with B2M, β-actin, and GAPDH acting as endogenous control genes.

Electron microscopy. EDL muscles from the control and denervated legs of young and aged animals were excised and cut at midbelly to obtain 2- to 3-mm serial sections. Muscle samples were incubated on ice for 1 h in 3.0% glutaraldehyde buffered with 0.1 M sodium cacodylate. Sections were washed three times in 0.1 M sodium cacodylate buffer and then postfixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate at room temperature. Muscle sections were dehydrated by washes with 30%, 50%, 80%, and 100% ethanol, then with ethanol-propylene oxide for 1 h, and finally with 60-nm sections were cut, collected on copper grids, and stained with uranyl acetate and lead citrate. Electron micrographs were obtained using a Philips EM201 electron microscope.

Statistical analyses. Values are means ± SE. Data were analyzed using a paired Student’s t-test or two-way repeated-measures ANOVA, as appropriate. Bonferroni’s post hoc test was applied to
Aged 543.7

tionally, epididymal adipose tissue mass was threefold greater parallel increase in heart weight (P were corrected for tibial length (data not shown). The aged animals These data were also replicated when muscle mass was cor-

rected for body mass (P result in significant decreases in TA muscle mass by 24%

P corrected for body weight (BW) The incorporation of gene products from the mitochondrial and nuclear genomes. Aged muscle exhibited a 29% reduction in mitochondrial content as reflected by COX activity compared with young muscle (Fig. 1B). After 7 days of denervation, mitochondrial content decreased by 50% and 34% (P < 0.05) in young and aged animals, respectively.

Autophagic protein expression. Whole muscle autophagic protein expression was evaluated for ULK1, Beclin1, ATG7, LC3II, and Parkin. Denervation resulted in marked increases of all proteins, with the exception of Parkin. These increases were particularly evident in young animals (P < 0.05; Fig. 2, A–D). ATG7 and Parkin protein expression were significantly increased in aged compared with young animals (Fig. 2, A, C, and D), while only ATG7 was further induced by denervation (Fig. 2C).

Localization of autophagic proteins to mitochondria. Denervation increased the mitochondrially localized expression of LC3II in the young and aged groups by 40–50% (P < 0.05; Fig. 2, D and E). Similarly, denervation significantly increased the presence of Parkin on mitochondria by seven- to eightfold (Fig. 2, D and F). The basal level of Parkin localization to mitochondria was considerably higher (P < 0.05) in the aged than the young animals. Thus the localization of these proteins to mitochondria, as induced by denervation, was attenuated in aged animals, although the levels achieved were higher (Fig. 2F) than those observed with denervation in the young animals.

Expression of p62 mRNA and protein. Denervation resulted in a significant increase in whole muscle p62 protein expression (Fig. 3, A and B). Similarly, denervation increased the localization of p62 to mitochondria for the young, but not the aged, animals (P < 0.05; Fig. 3, A and C). However, the basal level of p62 localization to mitochondria was significantly higher in the aged animals (Fig. 3C). Furthermore, basal mRNA expression of p62 was 3.5-fold higher in the aged animals (P < 0.05; Fig. 3D), while denervation significantly increased the mRNA expression of p62 in the young, but not the aged, animals (P < 0.05; Fig. 3D).

UPR. To evaluate the potential role of the UPR during denervation and aging, we measured the whole muscle expression of CHOP and BiP, two proteins that are essential for the UPR, as well as Xbp-1 mRNA, as an indicator of endoplasmic reticulum (ER)-associated protein degradation. CHOP protein expression was 17-fold higher in aged than young muscle and was further increased by 40% in response to denervation (P < 0.05; Fig. 4A). In the young animals, the increase in CHOP was 3.6-fold, but the absolute level attained was 7.6-fold lower than in the aged animals. In contrast, denervation resulted in a significant decrease in BiP protein expression in the young animals but had no effect in the aged animals, presumably because basal levels were lower with age (Fig. 4B). The mRNA expression of Xbp-1 was also elevated (P < 0.05) in aged

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Body Wt, g</th>
<th>Heart Wt, mg</th>
<th>Heart Wt/Body Wt, mg/g</th>
<th>Epididymal Fat Mass, g</th>
<th>Epididymal Fat Mass/Body Wt, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>354.8 ± 7.813</td>
<td>750 ± 0.03</td>
<td>2.37 ± 0.46</td>
<td>2.555 ± 0.14</td>
<td>8.92 ± 1.43</td>
</tr>
<tr>
<td>Aged</td>
<td>543.7 ± 18.57*</td>
<td>1,170 ± 0.05*</td>
<td>2.41 ± 0.45</td>
<td>8.704 ± 0.66*</td>
<td>15.58 ± 1.23*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8. *P < 0.05 vs. Young.
muscle and further increased as a consequence of denervation in the young and aged animals (Fig. 4C).

Whole muscle fission and fusion protein expression in young and aged muscle. Recent evidence indicates that mitochondrial fission and fusion may be among the key regulatory steps mediating mitochondrial degradation (37). Thus we measured the expression of four proteins that have been identified for their contribution to fission (Fis1 and Drp1) or fusion (Mfn2 and Opa1). The basal levels of Fis1 protein expression were increased by 2.5-fold in aged animals (P < 0.05; Fig. 4, D and E) and remained unaffected by denervation. Drp1 levels were not altered by age or denervation (Fig. 4D). Expression of Opa1 and Mfn2 was increased in aged animals, but only Mfn2 was further increased by denervation (P < 0.05; Fig. 4, D, F, and G).

Electron microscopy of young and aged muscle. Electron-microscopic analysis of muscle from the young and aged animals displayed the typical striated appearance common to skeletal muscle and an abundance of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. In support of the COX activity assessment of mitochondrial content, electron-microscopic images show evidence of a reduction in mitochondrial content following denervation, particularly in the SS region of the cells (Fig. 5B). Membranous structures encasing cellular material were also evident but were not apparent in normal, innervated muscle fibers. Muscle from the aged animals also exhibited reductions in SS and IMF mitochondria, along with several examples of mitochondrial cristae disruption (Fig. 5, A and C). Aged denervated muscle also revealed circular membranous structures encapsulating cellular content (Fig. 5, B and D), suggestive of the presence of autophagosomes.

Skeletal muscle lipofuscin content. The accumulation of lipofuscin within lysosomes is a well-documented adaptation that occurs in postmitotic tissues and is a distinct indicator of lysosomal dysfunction (13, 36). Lipofuscin granules were evident in aged control (Fig. 5G) and denervated (Fig. 5H) muscle, as well as young denervated (Fig. 5F), but not young control (Fig. 5E), muscle.

DISCUSSION

The decrements in cellular function as a consequence of aging have been linked with decreases in tissue performance and higher incidences of chronic disease (24). Sarcopenia is a prominent age-related change and is defined by a progressive loss in skeletal muscle mass and strength (17). This condition can be further exacerbated through chronic muscle inactivity, which is known to expedite atrophy and increase the incidence of frailty in the elderly (9). Although the molecular mechanisms mediating muscle atrophy in aged muscle are largely

Fig. 2. Denervation-induced alterations in autophagic protein expression in young and aged animals. A: typical immunoblots for ULK1, Beclin1, ATG7, and GAPDH in control (C) and denervated (D) muscles. B and C: quantification for ULK1 and ATG7. AU, arbitrary units. D: typical immunoblots for LC3II, Parkin, GAPDH, and mitochondrial (Mito) localization of LC3II and Parkin. E and F: total intensity (TI) of mitochondrial LC3II and Parkin. TI was used to measure mitochondrial extracts, because mitochondrial proteins are susceptible to changes in expression as a result of aging and disuse. Whole muscle protein analysis was corrected for loading with GAPDH. Values are means ± SE. *P < 0.05, main effect of denervation. †P < 0.05, main effect of age.
within these two groups. Our data have verified that aged muscle has a reduced ability to adapt to denervation and, thus, possesses a limited range of adaptive plasticity, a property of aged muscle that we previously observed in response to our experimental model of exercise (22). We expanded these conclusions by illustrating that the decreases in autophagy commonly associated with aging (23) may not necessarily be a result of a lack of autophagy gene expression but, rather, a consequence, at least in part, of an attenuated ability to up-regulate the autophagy response above an elevated baseline level as an adaptation to muscle disuse.

Denervation provides a useful method to study the role of the absence of contractile activity in maintaining muscle mass (1). We selected denervation as a model of muscle disuse to advance our understanding of skeletal muscle plasticity and to determine whether aged muscle possesses the same ability to undergo atrophy during this condition. Aged muscle exhibited the typical extent of sarcopenia relative to young muscle, and although the young and aged animals demonstrated significant reductions in muscle mass following denervation, the amount of atrophy experienced by aged muscle was attenuated compared with that experienced by young muscle. In addition to autophagy, changes in other pathways associated with denervation-induced muscle atrophy likely contribute to the atrophy. Although aged muscle has an enhanced sensitivity to mitochondrially mediated apoptosis (4, 8), other well-established catabolic pathways, such as the ubiquitin-proteasome pathway and autophagy, have been shown to decline with age (6, 23). Similar to this, mitochondrial content was significantly reduced in aged muscle following denervation; however, this decrease did not match the loss of mitochondria in the young animals. A 2:1 decrease in organelle content relative to muscle mass was noted in both age groups, suggesting a close relationship between muscle loss and mitochondrial degradation with denervation. However, mitochondria from aged muscle also demonstrated a disruption in cristae structure, and the presence of autophagosomes and lipofuscin is suggestive of impaired mitochondrial degradation. Thus a combination of factors, including fundamental decrements in the transcriptional activation of nuclear genes (22), as well as a possible impairment in the ability of the cell to effectively clear damaged mitochondria, likely led to the reduced mitochondrial content and function in aged muscle.

To obtain an overall view of the induction of the autophagy pathway in aged skeletal muscle, we assessed multiple markers of autophagy under basal conditions and in response to denervation. As noted above, we observed a significant, collective increase in basal autophagy protein (Fig. 6A) and mRNA (p62) expression in aged muscle. Increases in autophagy-related gene expression have been reported by others (42), although decreases have also been observed in aged muscle (11, 25). These discrepancies maybe a consequence of the fiber type distribution of the muscles selected, the small number of autophagy proteins analyzed in other studies, and the ages at which aged animals were selected for each study. In contrast, denervation induced a robust response in the expression of autophagy proteins in young muscle, whereas aged muscle demonstrated a much smaller induction of autophagy proteins (Fig. 6B). These data indicate that aged muscle possesses I an elevated expression of autophagy proteins, which could represent a compensatory response to a possible dysfunction in the final
execution of autophagy downstream of protein expression, and 2) a limited autophagic plasticity to chronic decreases in contractile activity. When these changes are related to muscle mass, it is interesting to note that the decay of almost half of the muscle mass (i.e., sarcopenia) in aged animals correlates with the twofold higher basal level of autophagy proteins (Fig. 6A) in aged muscle. Furthermore, the differential atrophy response to denervation was $\sim50\%$ higher in muscle from young animals, matching the $50\%$ greater induction of autophagy proteins (Fig. 6B) to the denervation stimulus. Thus there seems to be a direct relationship between the degree of atrophy and the level of proteins involved in the autophagy pathway. Of course, whether this parallels alterations in autophagic flux remains to be determined. Evidence for a possible disruption in the final execution of autophagy is provided by the significant accumulation of lipofuscin-positive lysosomes in aged control and denervated muscle. The formation of lipofuscin within lysosomes is common in aged tissues (13) and has been cited as an indicator of decreased lysosomal function and a rate-limiting step in mitophagy (3). Lipofuscin-positive lysosomes have also been observed in young muscle following exposure to high altitude (15); thus it is not unprecedented to detect their formation in young animals in response to muscle disuse.

The pronounced effects of aging on the physiological and biochemical properties of skeletal muscle include marked decrements in mitochondrial oxidative capacity (14) and an associated increase in the production of ROS (4, 39). In conjunction with age-related increases in the unfolded protein response (UPR) and mitochondrial dynamics proteins (Fig. 4), the results suggest that autophagy and its downstream effectors may be a key component in the adaptive response to muscle disuse and aging.
with this, ROS have been implicated in mediating the dissipation of $\Delta \Psi$, and they are a stimulus known to activate mitophagy (18, 32). Therefore, we assessed whether aging and denervation increased the mitochondrial localization of three well-established mediators of mitophagy, LC3II, Parkin, and p62. Indeed, the localization of p62 and Parkin with mitochondria was increased with age. This might be a further consequence of lipofuscin accumulation within lysosomes, resulting in a buildup of cellular debris (3, 13, 40). Interestingly, the localization of LC3II to mitochondria was increased in response to denervation for young and aged muscle. The increases in LC3II expression suggest that autophagosomal formation is likely not reduced in aged muscle and is supportive of the mitochondrial-lysosomal axis theory of aging (3). According to this theory, lipofuscin accumulation in lysosomes prevents the fusion of autophagosomes with lysosomes. Therefore, potential decrements in mitophagy within aged muscle, leading to an increase in mitochondria with respiratory dysfunction and accelerated ROS production in aged muscle (4, 22), are not a consequence of attenuated localization of LC3II.
Parkin, or p62 with mitochondria but could involve a decrease in lysosomal activity resulting from an accumulation of lipofuscin. Future studies will need to examine the possibility of mitophagy inhibition by examining the amount of autophagic flux within young and aged animals.

Our comprehension of mitophagy has been significantly improved by the discovery of regulatory processes not initially known for their participation in mitophagy. Several investigations have shown that mitochondrial dynamics and the UPR assist in identifying weakened mitochondria that need to be eliminated or have the ability to activate autophagy. Notably, it has been reported that the process of fission acts as a cellular mechanism to isolate dysfunctional mitochondrial segments, while fusion is a means to enable healthy mitochondria to return to the organelle reticulum (37). Interestingly, our fission protein analysis indicates an increase in Fis1 with age, suggesting the likelihood of a greater association of Fis1 with mitochondria. However, the profusion proteins Opal1 and Mfn2 were also elevated with age. This was an unexpected result, as others have shown an age-dependent decrease in Mfn2 mRNA expression (5), as well as decrements in mitochondrial fusion (33). Our data suggest that the cycle of fission and fusion may be elevated with age, providing substrates for mitophagy. However, steps beyond the formation of mitochondrial fragments probably limit the mitophagy process, as described above.

The UPR is a cellular mechanism that, when activated, has been shown to encode the expression of several autophagy proteins (44). Studies designed with the intent of elucidating how aging affects UPR-related proteins have shown increases in the expression of CHOP, an ER stress response protein that influences autophagy by decreasing the expression of Bcl-2 (35), and a decrease in BiP, an ER chaperone protein that inhibits UPR activity (29). Our UPR protein expression data support these conclusions, in that aging and denervation resulted in an increase in CHOP and a decrease in BiP expression. Moreover, Xbp-1 mRNA, a transcription factor known to be activated in response to ER stress, increased with age and in response to denervation. Together, these results suggest that the chronic effects of aging increase ER stress, which activates the UPR. Denervation produced a situation that further elevated the expression of CHOP and Xbp-1, while functioning to downregulate BiP. Interestingly, BiP has been identified as a critical regulator of autophagy in mammalian cells (21) and in motoneuron degradation (29). Certainly, a more detailed investigation is required to construct any definitive conclusions regarding how mitochondrial dynamics and the UPR affect muscle atrophy and mitochondrial turnover in young and aged muscle. In particular, it will be necessary to ascertain whether the increases in CHOP protein expression are directly influencing the UPR in mitochondria or in the ER.

In summary, our data indicate that the greater the level of the autophagy proteins, the more the muscle mass loss, either with age or as induced by denervation. Aged muscle exhibited an attenuated atrophy response to denervation, as well as the capacity to induce autophagic proteins, compared with muscle from young animals. In addition, evidence exists for an accumulation of mitochondria with impaired functional properties in aged muscle (8, 22). This may be a consequence of several factors, including 1) an impaired stress-induced signaling toward the transcription of autophagy proteins and/or 2) an accelerated feedback inhibition of mitophagic flux resulting from an age-associated decrease in lysosomal function, possibly induced by an accumulation of lipofuscin. These possibilities will be tested in future studies.

GRANTS
This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to D. A. Hood. D. A. Hood holds a Canada Research Chair in Cell Physiology.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.F.O., A.V., S.I., and O.O. performed the experiments; M.F.O. analyzed the data; M.F.O. and D.A.H. interpreted the results of the experiments; M.F.O. prepared the figures; M.F.O. drafted the manuscript; M.F.O. and D.A.H. edited and revised the manuscript; D.A.H. is responsible for conception and design of the research; D.A.H. approved the final version of the manuscript.

REFERENCES


