Denervation-induced mitochondrial dysfunction and autophagy in skeletal muscle of apoptosis-deficient animals

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Submitted 21 December 2011; accepted in final form 30 May 2012

O’Leary MFN, Vainshtein A, Carter HN, Zhang Y, Hood DA. Denervation-induced mitochondrial dysfunction and autophagy in skeletal muscle of apoptosis-deficient animals. Am J Physiol Cell Physiol 303: C447–C454, 2012. First published June 6, 2012; doi:10.1152/ajpcell.00451.2011.— Skeletal muscle undergoes remarkable adaptations in response to chronic decreases in contractile activity, such as a loss of muscle mass, decreases in both mitochondrial content and function, as well as the activation of apoptosis. Although these adaptations are well known, questions remain regarding the signaling pathways that mediated these changes. Autophagy is an organelle turnover pathway that could contribute to these adaptations. The purpose of this study was to determine whether denervation-induced muscle disuse would result in the activation of autophagy gene expression in both wild-type (WT) and Bax/Bak double knockout (DKO) animals, which display an attenuated apoptotic response. Denervation caused a reduction in muscle mass for WT and DKO animals; however, there was a 40% attenuation in muscle atrophy in DKO animals. Mitochondrial state 3 respiration was significantly reduced, and reactive oxygen species production was increased by two- to threefold in both WT and DKO animals.

Apoptotic markers, including cytosolic AIF and DNA fragmentation, were elevated in WT, but not in DKO animals following denervation. Autophagy proteins including LC3II, ULK1, ATG7, p62, and Beclin1 were increased similarly following denervation for both WT and DKO. Interestingly, denervation markedly increased the localization of LC3II to subsarcolemmal mitochondria, and this was more pronounced in the DKO animals. Thus denervation-induced muscle disuse activates both apoptotic and autophagic signaling pathways in muscle, and autophagic protein expression does not exhibit a compensatory increase in the presence of attenuated apoptosis. However, the absence of Bax and Bak may represent a potential signal to trigger mitophagy in muscle.

reactive oxygen species; muscle atrophy; mitochondria; mitophagy

MACROAUTOPHAGY (henceforth referred to as autophagy) is a highly conserved lysosomal-dependent degradation pathway that coordinates and oversees the digestion of organelles, proteins, and intracellular pathogens (4, 34, 42). More than 30 Autophagy-related (ATG) regulatory genes have been identified that are known to facilitate the engulfment of cytoplasmic material into double-membraned vesicles called autophagosomes (4, 42), and to assist in their degradation through fusion with lysosomes (10, 21). As a consequence, autophagy plays a prominent housekeeping role, which maintains homeostasis by selectively eliminating cellular debris. Moreover, although the activity of the autophagic pathway can be increased in response to stress stimuli, the importance of maintaining adequate levels of autophagy for cellular health and function is best demonstrated in several disease conditions, such as Parkinson’s and Pompe’s disease (27, 41), which are attributable, at least in part, to mutations in either ATG proteins or other autophagy-related genes.

Currently there is great interest in determining the meaningful purposes that autophagy has in regulating cellular health, and in more fully understanding the factors that direct selective forms of autophagy, such as mitophagy, which is autophagy-mediated mitochondrial turnover. Autophagy signaling is initiated in response to a multitude of factors including nutrient deprivation (12, 13, 25, 37) or oxidative stress (6, 18, 24, 31). Initiation of autophagy involves the activation of the unc-51 like kinase (ULK1)/ATG1 complex, which has been shown to be conserved in skeletal muscle (30), and the Beclin1/vacuole protein sorting/Pi3K complex, respectively. These operate in conjunction to mediate the initial assembly of the autophagosomal membrane (4, 19). The maturation and completion of the autophagosome is facilitated by a separate ubiquitin-like conjugation signaling cascade that culminates with the microtubule-associated protein 1 light chain 3 (MAP-LC3) incorporation of phosphatidylethanolamine into autophagosome membranes (4). Upon completion, the contents encased in mature autophagosomes are degraded by a battery of lysosomal hydrolases into macromolecules and then released into the cytoplasm (20, 21, 28).

Skeletal muscle is a postmitotic tissue that undergoes remarkable physiological and biochemical transformations in response to decreases in contractile activity, such as that brought about by chronic muscle disuse (2, 11). One well-established model that has been used to study the effects of disuse is denervation. Denervation produces a pronounced decrease in muscle mass (1, 23, 35), as well as accompanying reductions in capillary density (39) and mitochondrial content (1, 3, 40). Moreover, mitochondria may play an additional role during this disuse-induced muscle atrophy by mediating myonuclear apoptosis (1, 35). During this process several important proapoptotic factors, including Bax and Bak, are recruited to mitochondria where they can either hetero/homodimerize into a distinct apoptotic pore, or assist in the formation of other pores to facilitate the release of cytochrome c and AIF (1, 2, 7, 35). Previous investigations employing denervation have documented a significant increase in Bax mRNA (35) and protein expression (1, 23), as well as a decrease in the expression of Bcl-2, an antiapoptotic factor known for its ability to neutralize Bax (1). As a consequence of this, the rate of mitochondrial transition pore formation has been shown to increase following denervation (1). These denervation-induced responses contribute to a significant increase in caspase-3 cleavage (26, 35) and myonuclear DNA fragmentation (1, 16, 23, 35). Despite this robust activation of apoptosis during disuse atrophy, it is well recognized that the increases in apoptosis are only able to

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account for a fraction of the atrophy observed during disuse. Moreover, apoptosis and its associated signaling do not explain the intracellular remodeling that occurs during denervation, particularly related to the dramatic increase in mitochondrial degradation. Thus autophagy is of significant interest because it not only provides a means of selective mitochondrial turnover that has rarely been studied in this denervation model, but it could also be involved as an influential signaling pathway in muscles that are apoptosis deficient. In support of this, early research identified the presence autophagosomes in denervated skeletal muscle (32), whereas more recent work has demonstrated that muscle denervation not only induces an increase in several genes related to both the autophagic and lysosomal pathways (15, 17) but also acts upon important transcriptional regulators of these pathways, such as FoxO3 (5, 15). However, the relevance of autophagy protein expression and its contributions to selective forms of autophagy, such as mitophagy, have yet to be elucidated in skeletal muscle. Thus the purpose of this study was to specifically examine the effect of denervation on autophagy signaling pathways in wild-type (WT; C57BL/6) and apoptotic deficient (Bax/Bak double knockout [DKO]) mice. We hypothesized that autophagy signaling cascades and gene expression would be elevated in denervated skeletal muscle and that autophagy would exhibit a compensatory response in view of the attenuated rate of apoptosis in Bax/Bak knockout animals. Our data show that chronic denervation of skeletal muscle is a powerful activator of ATG gene expression and that markers of mitophagy are increased, representing a pathway of mitochondrial clearance during chronic muscle disuse.

MATERIALS AND METHODS

Animal surgery. C57BL/6 (WT) and B6:129-Bax<sup>+/−<sup>/Thyrb/J (DKO) mice (n = 60; 3 mo of age; 006329; Jackson Laboratory) were housed in pairs and given food and water ad libitum. Denervation surgery was performed as described previously (1, 23). Denervation surgery was performed as previously described (1, 23). The surgical procedures were conducted in accordance with the regulations of the York University Animal Care Committee and were approved by the committee. Briefly, animals were anesthetized using xylazine and ketamine (0.2 ml/g body wt). Under sterile conditions a 1- to 2-cm incision was made and a 0.25-cm portion of the sciatic nerve was excised. Thus the tibialis anterior (TA) and gastrocnemius (GASTROC) muscles were extracted and used for mitochondrial analysis. A volume of 50 μl of mitochondrial sample was added to 250 μl of VO<sub>2</sub> buffer containing 250 mM sucrose, 50 mM KCl, 10 mM Tris-HCl, 10 mM K<sub>2</sub>HPO<sub>4</sub>, and 0.2% BSA (pH 7.4). Respiration was measured in the presence of 1) 11 mM glutamate (state 4 respiration) and 2) 0.4 mM ADP (state 3 respiration) as done previously (1, 23).

ROS production. ROS production was measured in isolated SS mitochondria. This assay was designed to evaluate total ROS production, which includes various forms of ROS such as hydrogen peroxide and peroxynitrite. Briefly, mitochondria (50 μg) were incubated with VO<sub>2</sub> buffer and 50 μM dichlorodihydrofluorescein diacetate in a black polystyrene 96-well plate. ROS production was assessed during state 4 respiration by adding 10 mM glutamate and 70 mM ADP immediately before the addition of dichlorodihydrofluorescein diacetate. ROS production was measured with a Synergy HT microplate reader, and the data were compiled with KC4 (v3.0) software.

Protein release assay. Isolated SS mitochondria were incubated with mitochondrial resuspension medium containing 2 mM of FeSO<sub>4</sub>, 75 μM of H<sub>2</sub>O<sub>2</sub>, and 10 mM of malate. Incubations occurred for 5 min at 30°C. Reaction mixtures were then centrifuged at 14,000 g (4°C) to pellet mitochondria, and the supernatant fraction was collected for subsequent measurement of cytochrome c release using Western blot analysis.

Immunoblotting. Whole muscle protein extracts, isolated mitochondria, and whole muscle cytosolic extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes with a wet electrotransfer apparatus (165–8000; Bio-Rad). Nitrocellulose membranes were blocked in a 5% skim milk solution containing 1× Tris-buffered saline-Tween (TBST) 20, 25 mM Tris-HCl (pH 7.5), 1 mM NaCl, and 0.1% Tween 20. Membranes were then incubated overnight at 4°C with the appropriate concentration of primary antibody LC3II (1:500; Cell Signaling), Beclin1 (1:500; Cell Signaling), p62 (1:200; Sigma), ATG7 (1:3,000; Sigma), ULK1 (1:500; Sigma), and AIF (1:500; Santa Cruz). Membranes were subsequently washed in TBST to remove any excess primary antibody and then incubated with the appropriate secondary antibody at room temperature for 60 min. Membranes were then washed in TBST and developed with the enhanced chemiluminescence method. Films were scanned and quantified using SigmaScan Pro (version 5) software. To control for loading, protein quantifications were corrected with GAPDH immunoblotting (1:30,000; ab8245; Abcam,). Our data indicate that there was no effect of denervation on the expression of GAPDH over the time course of the experimental design used in this study.

ELISA cell death assay. TA muscles were extracted, blotted to remove excess liquid, minced, and homogenized. Differential centrifugation was then used to isolate cytosolic extracts and protease inhibitors were added, as described previously (29), to prevent protein degradation. Cell death assays were conducted using the ELISA Cell Death Detection Kit (1774425; Roche Applied Science) and were performed as described previously (38). Briefly, DNA fragmentation was measured in cytosolic fractions with a Synergy HT microplate reader, and the data were compiled with KC4 (v3.0) software.

Total RNA isolation. Total RNA was isolated using TRIzol reagent (15590-026; Invitrogen,) as described previously (14) 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 the 28S and 18S rRNA on denaturing formaldehyde-1% agarose gels.

RT-PCR. Total RNA (2 μg) was reverse transcribed to cDNA using RT<sup>2</sup> First Strand Kit as recommended by the manufacturer (330451; SABiosciences).

PCR arrays. The gene expression of 84 key autophagic genes were profiled by RT<sup>2</sup> Profiler Autophagy PCR Arrays (SABioscience, PAMM-084) as recommended by manufacturer. RT-PCRs were performed in 96-well plate format using the ABI 7500 FAST Real-Time PCR System. Fold changes in autophagic gene expression from denervated samples relative to control samples were calculated using the ΔΔC<sub>T</sub> method using the integrated software package for PCR Array Systems provided by the manufacturer (RT<sup>2</sup> Profiler PCR Array Data Analysis Template v3.3). ΔΔC<sub>T</sub> values from each sample were

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normalized by three housekeeping genes that did not change across the conditions (APP, ATG16L, ATG4b).

Statistical analysis. Data were expressed as means ± SE. Two-way repeated-measures ANOVA was used to determine whether there was a main effect of denervation and/or an effect of genotype. A Bonferroni post hoc test was applied to detect differences within groups. Data were considered statistically different if \( P < 0.05 \).

RESULTS

Denervation-induced changes in muscle mass. Denervation-induced muscle disuse resulted in a 16% and 11% decrease in WT and DKO TA muscle mass, respectively (\( P < 0.001 \); Fig 1A). However, when these data were expressed as a percentage of control values there was a 40% attenuation of muscle atrophy in the DKO animals following just 7 days of denervation (\( P < 0.001 \); Fig. 1A, inset) compared with the WT animals.

**SS mitochondrial function and muscle oxidative stress.** Denervation decreased SS mitochondrial state 3 (active) oxygen consumption in both WT and DKO animals (\( P < 0.05 \); Fig. 1B), whereas no significant decreases in SS mitochondrial state 4 (passive) oxygen consumption were observed (Fig. 1C). Decreases in mitochondrial electron transport chain function are usually associated with elevations in ROS production. Indeed, state 3 SS mitochondrial ROS production was increased by 2.9- and 2.2-fold in WT and DKO animals, respectively (\( P < 0.001 \); Fig 2A). Additionally, ROS production was also markedly elevated during state 4 respiration in both denervated groups (\( P < 0.01 \); Fig. 2B).

Denervation-induced mitochondrial mediated apoptosis. An important marker of denervation-induced apoptotic signaling is the release of proteins through mitochondrial transition pores (1). Our study shows a main effect of denervation and an ~1.9-fold increase in cytosolic AIF protein expression (\( P < 0.01 \); Fig. 3A). To examine the downstream consequence of this, we measured the extent of DNA fragmentation. After 7 days of denervation, WT animals exhibited a 40% increase in DNA fragmentation, whereas no significant change in DNA

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![Fig. 1. Tibialis anterior (TA) muscle mass and subsarcolemmal (SS) mitochondrial respiration following 7 days of denervation.](image1.png)

**Fig. 1.** Tibialis anterior (TA) muscle mass and subsarcolemmal (SS) mitochondrial respiration following 7 days of denervation. A: denervation (Den)-induced decreases in TA muscle mass are shown as a percentage of control (Con; inset) and corrected for body mass (\( n = 20 \)). B and C: oxygen consumption in SS mitochondria during state 3 and state 4 respiration, respectively (\( n = 6 \)). White columns represent control muscles; black columns represent 7-day denervated muscles. Values are means ± SE. *\( P < 0.05 \), main effect of denervation. WT, wild-type; DKO, Bax/Bak double knockout.

![Fig. 2. Reactive oxygen species (ROS) production in SS mitochondria isolated from muscle.](image2.png)

**Fig. 2.** Reactive oxygen species (ROS) production in SS mitochondria isolated from muscle. A: SS ROS production during state 3 and state 4 mitochondrial respiration, respectively (\( n = 6 \)). White columns represent control muscles (Con); black columns represent 7-day denervated muscles (Den). Values are means ± SE. *\( P < 0.05 \), main effect of denervation.
fragmentation was detected in DKO animals (\(P < 0.01\); Fig. 3B). This difference is likely a result of a greatly attenuated ability of mitochondria isolated from DKO animals to release cytochrome c (Fig. 3B, inset).

Autophagic protein expression following denervation. Autophagy protein expression was assessed to investigate whether this molecular signaling pathway was sensitive to decreases in contractile activity. Whole muscle LC3II expression was increased by 50-fold following denervation in both WT and DKO mice (\(P < 0.001\); Fig. 4A). This was accompanied by an increased LC3II localization to SS mitochondria from DKO animals, which was more pronounced than in WT animals (\(P < 0.05\); Fig. 4B). Several other important mediators of autophagy were also increased with 7 days of denervation. Whole muscle ULK1, beclin-1, ATG7, and p62 was significantly elevated by 1.40- to 2.2-fold in denervated muscle (\(P < 0.001\); Fig. 5A-C). It is interesting that there was no significant difference in the expression of these autophagy proteins in response to denervation between the WT and DKO animals.

PCR array of autophagic gene expression following denervation. To further investigate whether autophagy was activated in response to decreases in contractile activity, we assessed the mRNA expression of an array of several important autophagy genes in WT animals. The expression of beclin-1, LC3II, ATG7, and p62 was significantly elevated by 1.40- to 2.2-fold in denervated muscle (\(P < 0.001\); Fig. 5D). It is interesting that there was no significant difference in the expression of these autophagy proteins in response to denervation between the WT and DKO animals.
2.20-fold ($P < 0.05$; Fig. 6), whereas ULK1 mRNA expression was not significantly altered (Fig. 6). Furthermore, the mRNA levels of other essential autophagy genes such as p53, Gabarapl2, Gabarap, caspase-3, and Bid were induced by 1.4- to 37-fold following denervation ($P < 0.05$; Fig. 6).

**DISCUSSION**

Chronic disuse of skeletal muscle results in cellular atrophy that is paralleled by a commensurate decrease in maximal force production and endurance performance (9). However, the underlying mechanisms that are selectively activated by disuse, and the exact contribution that different signaling pathways have in producing muscle atrophy, are not yet fully understood. Several studies have examined the influence of denervation on apoptotic signaling pathways in muscle and have observed an increase in the expression of apoptotic proteins, as well as greater DNA fragmentation (1, 23, 35). Thus, apoptosis appears to contribute to muscle atrophy during disuse conditions. Yet, the induction of apoptosis can only account for a fraction of the total atrophy that occurs during disuse and, more importantly, cannot account for several other atrophy-related adaptations. In particular, the decline in mitochondrial content per gram of muscle, a major influence on muscular endurance capacity, must be a result of processes, which are independent of apoptotic signaling, such as autophagy. Although there have been reports that markers of autophagy are increased in denervated muscle (15, 17, 23, 24, 32, 43) many questions still exist with regard to the functional role that this pathway has during muscle atrophy. Therefore, the main purpose of this study was to investigate whether the extent of the denervation-induced increase in autophagy was upregulated in animals with an attenuated apoptotic pathway in the absence of Bax and Bak.

Atrophy resulting from denervation is known to proceed in a biphasic manner that begins with a rapid loss in muscle mass over the first 14 days. It is during the initial rapid phase that the vast majority of muscle atrophy occurs in...
Fig. 6. Real-time PCR array of autophagic gene expression in WT control and denervated TA muscles (n = 6). Dark gray columns represent autophagy genes that are significantly (P < 0.05) increased following denervation, and light gray columns represent autophagy genes that were not significantly changed as a result of denervation. The white column represents autophagy genes that had a significant decrease in their expression following denervation (P < 0.05).

conjunction with reductions in mitochondrial content and function (40), along with increases in apoptotic protein expression (1, 23, 35). Accordingly, we chose 7 days of denervation to capture the majority of the signaling events associated with atrophy. Our data demonstrate that regular contractile activity is an indispensable stimulus that is required for the maintenance of muscle mass in both WT and DKO animals, as both groups experienced muscle atrophy in response to denervation. However, the absence of the proapoptotic proteins Bax and Bak reduced apoptotic signaling and DNA fragmentation. DKO animals showed a significantly lower cytosolic AIF and cytochrome c release when compared with WT animals. Hence, the loss of Bax and Bak potentially limits the formation mitochondrial apoptotic pores and inhibits the release of several proapoptotic proteins, including AIF and cytochrome c. As a consequence, the induction of downstream apoptotic signaling cascades in denervated muscle from DKO animals would be decreased, and this could, in part, explain the reduced DNA fragmentation as well as the attenuated denervation-induced muscle atrophy observed in these mice. Taken together, these data clearly indicate that mitochondrially mediated apoptosis via Bax and Bak signaling is involved in mediating a portion of the muscle atrophy evident during denervation. Our data extend the previous findings of Siu and Alway (36) who showed that denervation-induced muscle atrophy can be modestly attenuated in the absence of Bax expression. It is interesting that although the loss of Bax and Bak had an impact on mitochondrial protein release signaling, it did not have an effect on either the decrease in mitochondrial function or the accelerated production of ROS resulting from denervation. In addition, despite the reduction in DNA fragmentation in DKO muscle, these animals still exhibited a decrease in muscle mass, which clearly points to the involvement of alternative catabolic pathways (e.g., ubiquitin-proteasome system) (43) during denervation-induced muscle atrophy.

Our next question was whether the autophagic response was activated in denervated muscle in an accelerated manner to compensate for the diminished apoptotic signaling in DKO animals. Thus we evaluated the expression of several proteins from distinct parts of the autophagy pathway following denervation. Whole muscle ULK1, beclin-1, and ATG7 protein expression were all increased following denervation, signifying an enhanced ability for disused muscle to sequester and begin to build the incipient autophagosome. The expression of many of these genes was also elevated at the mRNA level, suggesting that denervation activates autophagy signaling pathways at the level of transcription and/or mRNA stability. One of the most dramatic changes in response to denervation was evident from the 50-fold increase in whole muscle LC3II protein expression. This was also accompanied by a greater localization of LC3II with mitochondria, as well as an increase in whole muscle p62 protein and mRNA expression. Thus it is apparent that denervation induces an increase in autophagic genes and that the rapid turnover of mitochondria during disuse can be attributed, at least in part, to an enhancement in mitophagy signaling. Interestingly, the loss of the proapoptotic proteins Bax and Bak did not have an additive effect on the expression of autophagy proteins, although the localization of LC3 to mitochondria was markedly elevated in DKO animals. The mechanism involved in this enhancement of mitophagy signaling in the absence of Bax/Bak remains to be resolved and will be further confirmed by additional measures of mitophagy such as Parkin translocation to mitochondria, PINK-1 stabilization, and mitochondrial protein ubiquitination.

A question that emerges from our results is whether the accelerated autophagic response is sufficient to maintain muscle function in response to chronic muscle disuse, such as that imposed by denervation. Indirect evidence that the augmentation in autophagy and mitophagy signaling remains insufficient is provided by the continuing presence of dysfunctional mitochondria, exemplified by marked reductions in state 3 respiration. Mitochondrial dysfunction such as this produced by chronic muscle disuse may have a number of causes, including 1) a decrease in oxidative protein expression (3, 33), 2) reductions in mitochondrial protein import (33), or 3) insufficient mitophagy. Regardless of the mechanism, this decrement in mitochondrial function is directly associated with the twofold increase in ROS production, which is a potent trigger for both mitochondrially mediated apoptosis as well as autophagy pro-
tein expression (8, 22). Consequently, the similar increases in autophagy proteins following denervation in WT and DKO animals are likely attributable to the comparable increases in ROS production. Thus our data implicate ROS as one of the major driving forces for autophagy in response to muscle denervation. In addition, it is evident that the attenuated loss of muscle mass observed in our DKO animals is not influenced by any differences in the autophagic response between the WT and DKO animals.

In summary, our data indicate that autophagy signaling is rapidly accelerated during muscle denervation, but suggest that this increase is insufficient to maintain mitochondrial function. In addition, we found no evidence for a compensatory increase in autophagic protein expression in the presence of attenuated apoptotic potential, although a tendency for augmented mitophagy was observed. This suggests that the absence of Bax and Bak within mitochondria represents an additional signal for mitochondrial clearance via the mitophagy pathway.

ACKNOWLEDGMENTS

D. A. Hood holds a Canada Research Chair in Cell Physiology.

GRANTS

This work was supported by a Natural Sciences and Engineering Research Council (NSERC) of Canada grant (to D. A. Hood).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.F.O., and D.A.H. edited and revised manuscript; D.A.H. conceived and interpreted results of experiments; M.F.O. prepared figures; M.F.O. drafted manuscript; M.F.O. and D.A.H. approved final version of manuscript.

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