Effect of denervation-induced muscle disuse on mitochondrial protein import

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Singh K, Hood DA. Effect of denervation-induced muscle disuse on mitochondrial protein import. Am J Physiol Cell Physiol 300: C138–C145, 2011. First published October 13, 2010; doi:10.1152/ajpcell.00181.2010.—This study determined whether muscle disuse affects mitochondrial protein import and whether changes in protein import are related to mitochondrial content and function. Protein import was measured using a model of unilateral peroneal nerve denervation in rats for 3 (n = 10), 7 (n = 12), or 14 (n = 14) days. We compared the import of preproteins into the matrix of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria isolated from the denervated and the contralateral control tibialis anterior muscles. Denervation led to 50% and 29% reductions in mitochondrial protein import after 14 days of disuse in SS and IMF mitochondria, respectively. This was accompanied by significant decreases in mitochondrial respiration and reactive oxygen species (ROS) production. Denervation significantly reduced the expression of translocases localized in the inner membrane (Tim23), outer membrane (Tom20), and mitochondrial heat shock protein 70 (mtHsp70), especially in the SS subfraction. Denervation also resulted in elevated ROS generation, and exogenous heat shock protein 70 (mtHsp70) motor complex in an ATP-dependent fashion (39).

Method

Chronic muscle disuse results in biochemical alterations that affect the endurance performance of muscle, which is particularly dependent on mitochondrial content (4, 20, 31). Skeletal muscle mitochondrial content is determined by the net synthesis and degradation of organellar proteins. Muscle disuse accelerates the rate of protein degradation, with concomitant decreases in the synthesis of mitochondrial proteins (29). The principle pathway for mitochondrial assembly involves the import of newly synthesized preproteins from the cytosol into existing mitochondria (7, 21, 26). These preproteins are actively transported into existing organelles by the protein import machinery (PIM). The important components of the import pathway include the translocases, which aid in the movement of preproteins across membranes (16). These translocases are localized in the outer membrane (TOM complex) or the inner membrane (TIM complex). During mitochondrial import, Tom20 is important for the recognition and binding of cytosolic preproteins possessing targeting presequences, as well as for transferring them into the matrix via the protein import pore (17). Matrix-destined proteins have to be channeled through Tim23 (5, 40), and are actively pulled into the matrix by the mitochondrial heat shock protein 70 (mtHsp70) motor complex in an ATP-dependent fashion (39).

1 Experimental design. Normal male Sprague-Dawley rats (300–400 g) were randomly assigned to one of three time-course groups: 3 (n = 10), 7 (n = 12), and 14 days (n = 14). The rats underwent surgery to induce unilateral denervation, after which the animals were allowed to recover for either 3, 7, or 14 days. At each time point, animals were

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euthanized, and SS and IMF mitochondria were isolated from the denervated and contralateral nondenervated limb muscles for comparisons of organellar function (i.e., ROS production, respiration), content as assessed by cytochrome c oxidase (COX) activity, protein import, and the expression of protein import machinery components. Resulting changes in mitochondrial respiration or content were correlated with protein import to determine potential relationships between these parameters.

In vivo denervation protocol. Rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (0.2 ml/100 g of body wt). The fast-twitch tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were denervated by exposing the lateral common peroneal nerve and excising a 5-mm section. The incision was sutured after administration of sterile ampicillin. The contralateral leg served as an internal control for each animal. After surgery, the skin was closed with metal clips, and the day of surgery represented day 0 of the time course. Denervated animals were given amoxicillin in their drinking water for 1 wk after surgery (0.025% wt/vol). All procedures involving animals were approved by the York University Animal Care Committee, in accordance with the Canadian Council on Animal Care.

Tissue extraction and mitochondrial isolation. Animals were anesthetized, and both denervated and contralateral TA muscles were excised, minced, and briefly homogenized using an Ultra-Turrax polytron at 40% power output. The IMF and SS mitochondrial subfractions were isolated by differential centrifugation as described previously (10). Freshly isolated mitochondria were used for protein import, respiration, and ROS emission assays. Aliquots of each mitochondrial sample were also frozen for future use in immunoblotting assays. The EDL muscles were clamp frozen in liquid nitrogen and used for COX activity measurements.

COX activity. Pulverized EDL muscles were diluted in muscle extraction buffer (in mM: 100 KH2PO4, 100 Na2HPO4, and 2 EDTA, pH 7.2) and sonicated 3× for 5 s on ice. Supernatant fractions were added to a test solution containing fully reduced cytochrome c, and this reaction was carried out within a 96-well plate at 30°C using a Synergy-HT microplate reader. COX activity was assessed by the maximal rate of reduction in absorbance at 550 nm.

In vitro transcription, translation, and import of OCT. The full-length cDNA clone for ornithine transcarbamylase (OCT) was a generous gift from Dr. Gordon Shore (McGill University, Montreal, Canada). This cDNA was transcribed and translated in vitro as previously described (12). Briefly, the vector containing the OCT cDNA was linearized using SacI and subsequently phenol extracted and ethanol precipitated. OCT was in vitro transcribed at 40°C for 90 min using SP6 RNA polymerase, followed by in vitro translation in the presence of [35S]methionine within a rabbit reticulocyte lysate system.

The mitochondrial protein import assay was performed as outlined previously (34, 38). Briefly, mitochondria were preincubated for 10 min at 30°C before the import assay. For experiments investigating the effect of ROS on protein import, the appropriate volume of H2O2 was added to the reaction so that the final concentration of H2O2 was either 5 or 10 μM. For 0 μM of ROS, H2O2 was substituted with water. Translation mix (12 μl per 25 μg of mitochondrial protein) was added to the mitochondrial import incubation to proceed at 30°C for 20 min. After 20 min, import was halted by the addition of an aliquot of the mitochondrial translation mix to an ice-cold sucrose cushion (in mM: 600 sucrose, 100 KCl, 20 HEPES, and 2 MgCl2). Mitochondria were pelleted by centrifugation for 15 min at 16,000 g (4°C) and resuspended in 20 μl of ice-cold breaking buffer (600 mM sorbitol, 20 mM HEPES, pH 7.4). The samples were then denatured at 95°C in the presence of lysis buffer for 5 min, quick cooled on ice, and then resolved through a 12% SDS-polyacrylamide gel. Gels were processed and dried as described elsewhere (12, 34). Images and subsequent quantification were obtained with electronic autoradiography (Quantity One, Bio-Rad, Hercules, CA). Imported mature OCT was distinguished from precursor OCT because of its lower molecular weight. The percentage of available protein that was imported was calculated based on the ratio of the intensity of the mature OCT and total OCT (sum of mature and precursor bands).

Mitochondrial respiration assay. Fifty microliters of isolated mitochondrial samples were incubated with 250 μl of VO2 buffer (in mM: 250 sucrose, 50 KCl, 25 Tris, and 10 K2HPO4, pH 7.4) in a Clark oxygen electrode respiratory chamber (Strathkelvin Instruments, North Lanarkshire, Scotland) with continuous stirring at 30°C. Mitochondrial oxygen consumption was measured in the presence of exogenously added 10 mM glutamate to assess state 4 respiration, followed by 0.44 mM ADP to elicit state 3 respiration. In addition, NADH was added during state 3 measurements to evaluate the integrity of the inner mitochondrial membrane.

Mitochondrial ROS production. SS and IMF mitochondria (75 μg) were incubated with 50 μM dichlorodihydrofluorescein diacetate (H2DCFDA) and VO2 buffer at 37°C for 30 min in a 96-well plate. ROS production is directly proportional to fluorescence emission between 480 and 520 nm measured using a multidetection microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT). ROS emission was assessed during state 4 and state 3 respiration, and the microplate data were compiled and analyzed using KC4 (version 3.0).

Immunoblotting. Isolated SS and IMF mitochondrial protein extracts were separated by performing 15% SDS-PAGE and subsequently electroblotted onto nitrocellulose membranes. After transfer, membranes were blocked for 1 h in 1× TBST containing 5% skim milk. Blots were then incubated in blocking buffer with antibodies detected against Tim23 (1:500), Tom20 (1:500), mtHSP70 (1:1,000), and ANT (1:2,000) overnight at 4°C. Antibodies were obtained from Santa Cruz (Tom20, sc-11415), BD Biosciences (Tim23, 611222), and Assay Designs (mtHSP70, SPS-825F). The ANT antibody was generously provided by Dr. K. B. Freeman (McMaster University, Hamilton, Ontario). After three washes for 5 min, blots were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase and washed again three times for 5 min each. Antibody-bound protein was revealed using the ECL method. Films were scanned and analyzed using SigmaScan Pro 5.0 software (Aspire Software, Ashburn, VA).

Statistical analysis. Data were expressed as means ± SE. Paired Student’s t-tests were used for comparisons between IMF and SS mitochondria isolated from control muscle. A one-way or two-way analysis of variance was used to assess all time-course groups, followed by Bonferroni’s post hoc test to assess differences within groups. To evaluate the relationship between changes in mitochondrial content and protein import, as well as protein import and mitochondrial respiration rates, a Pearson’s correlation coefficient (r) was calculated and reported for all significant relationships. Statistical differences were considered significant if P < 0.05.

RESULTS

Basal differences in function and protein import within control SS and IMF mitochondrial subfractions. State 3 respiration was 2.5-fold greater in IMF mitochondria (P < 0.05; Fig. 1A). In contrast, ROS production during state 3 respiration was 4.3-fold higher in SS mitochondria when compared with the IMF subfraction (P < 0.05; Fig. 1B). Precursor OCT (pOCT) import was twofold greater in IMF mitochondria than those observed in the SS mitochondrial subfraction (P < 0.05; Fig. 1C). mtHSP70 protein levels were 30% lower in IMF mitochondria when compared with the SS subfraction (P < 0.05; Fig. 1D), whereas levels of Tim23 and Tom20 expression were not different between IMF and SS mitochondria (data not shown).

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Effect of denervation on muscle mass and mitochondrial content. Denervation of the TA muscle resulted in significant reductions of muscle mass by 7, 33, and 65% after 3, 7, and 14 days, respectively, compared with control muscle (P < 0.05; Fig. 2A). State 4 respiration was not affected by denervation in IMF mitochondria but was decreased by 17% and 26% at 3 and 7 days, respectively, in SS mitochondria (P < 0.05; Fig. 2B).

Effect of denervation on mitochondrial respiration. Mitochondrial ROS production was assessed in SS and IMF subfractions during state 3 and state 4 respiration. State 3 ROS production was increased by 3.7-fold in SS mitochondria after 14 days of denervation, whereas IMF mitochondrial generated 4.2-, 2.8-, and 5.5-fold higher levels of ROS after 3, 7, and 14 days, respectively (P < 0.05; Fig. 3A). State 4 ROS emission in SS mitochondria was elevated by 1.8- and 3.9-fold following 7 and 14 days, respectively. Finally, IMF mitochondrial state 4 ROS production was enhanced by 1.9- and 2.9-fold after 3 and 14 days of denervation (P < 0.05; Fig. 3B).

Effect of denervation on mitochondrial protein import. OCT import was blunted in both the SS and IMF subfractions during all three time periods of denervation. Protein import in SS mitochondria from denervated muscle was reduced by 29, 42, and 50%, whereas IMF mitochondrial protein import was reduced by 13, 19, and 29% at 3, 7, and 14 days, respectively (P < 0.05; Fig. 5).

Effect of denervation on mitochondrial protein import machinery expression. Corresponding to the deceleration of OCT import in denervated muscle, there was a decrease in the protein expression of mitochondrial import machinery components, Tim23, Tom20, and mtHSP70. Tim23 levels were reduced by 40% following 14 days of denervation in IMF mitochondria.

Fig. 1. Differences in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial function and protein import. A: oxygen consumption during ADP stimulated respiration (state 3). B: reactive oxygen species (ROS) production during state 3 respiration. C: rate of precursor ornithine transcarbamylase (pOCT) protein import. A representative autoradiograph is shown. D: expression of mitochondrial heat shock protein 70 (mtHSP70) protein levels. A typical Western blot is illustrated (n = 23–26). *P < 0.05 SS vs. IMF. Values are means ± SE.

Fig. 2. Effect of 3, 7, and 14 days of denervation on muscle atrophy and mitochondrial content. A: tibialis anterior (TA) muscle mass corrected for body weight (BW; n = 10–14 per group). B: cytochrome c oxidase (COX) activity in response to different periods of denervation (n = 8–10 per group). *P < 0.05 control vs. denervated at each time point; †P < 0.05 vs. 3 days; ‡P < 0.05 vs. 7 days. Values are means ± SE.
mitochondria, whereas Tim23 expression in SS mitochondria was decreased by 45% and 21% after 3 and 14 days, respectively (P < 0.05; Fig. 6). In contrast, Tom20 was not affected by denervation in IMF mitochondria but was reduced by 33% after 14 days in SS mitochondria (P < 0.05). mtHSP70 was decreased in SS mitochondria by 49% and 66% following 3 and 14 days, respectively, whereas IMF mitochondrial mtHSP70 protein levels declined by 37% and 68% at the same time in IMF mitochondria (P < 0.05; Fig. 6).

Assessment of ROS on protein import. To investigate the effect of ROS on protein import, we incubated mitochondria and OCT preprotein in the presence of either 5 or 10 μM hydrogen peroxide. Protein import in SS and IMF mitochondria was reduced in comparable fashion by 35% and 65% following 3 and 14 days, respectively, whereas IMF mitochondrial mtHSP70 protein levels declined by 37% and 68% at the same time in IMF mitochondria (P < 0.05; Fig. 7).

Correlational analysis among mitochondrial content, protein import, and respiration. A significant correlation (r = 0.69) between changes in COX activity and differences in SS mitochondrial OCT import (P < 0.05; Fig. 8A) was observed. Additionally, there was a high correlation (r = 0.95) between protein import and mitochondrial state 3 respiration (P < 0.05; Fig. 8B).

DISCUSSION

A hallmark feature of skeletal muscle disuse-induced atrophy is a decrease in mitochondrial biogenesis (3, 36). Our previous work has characterized reduced muscle performance and mitochondrial enzyme activity (41), altered blood flow (15), and increased mitochrondrially mediated cell death during denervation (3, 27, 28). The majority of mitochondrial proteins are nuclear-encoded, and mitochondrial biogenesis requires the protein import machinery to transport these cytosolic proteins into the organelle (7, 21). Our previous work has illustrated the adaptability of this pathway in skeletal muscle mitochondria responding to various physiological perturbations such as chronic contractile activity (18, 22, 37), aging (13, 22), thyroid hormone treatment (11, 12, 34), and muscle differentiation (19). However, the adaptation of the protein import system in response to chronic muscle disuse has never been previously investigated. In addition, skeletal muscle comprise two subpopulations of mitochondria, SS and IMF, which are found in different cellular compartments, possessing subtle differences in functional and biochemical properties. These characteristics could be accounted for, in part, by divergent rates of protein import within the two subfractions (20). Thus the main purposes of the present study were 1) to evaluate denervation-induced adaptations in protein import within skeletal muscle SS and IMF mitochondria, and 2) to relate these changes to alterations in the expression of import machinery components and mitochondrial function.

First, we wanted to relate any basal differences in SS and IMF mitochondrial protein import to organelle function and the expression of import machinery components. Current results confirm higher rates of state 3 respiration along with lower ROS generation in IMF when compared with SS mitochondria, in agreement with previously reported values (Fig. 1, A and B) (3, 28, 41). Enhanced ETC activity in IMF mitochondria was accompanied by a twofold higher rate of OCT import in the IMF subfraction (Fig. 1C). We also measured the protein expression of Tim23, Tom20, and mtHsp70, which are critical components of the import machinery governing matrix-des-
tined proteins (8). Although, we found no differences in SS and IMF mitochondrial Tom20 and Tim23 content (data not shown), mtHsp70 protein levels were significantly higher in the SS subfraction (Fig. 1D), a finding that is similar to our previous observations (18, 19, 38). Thus it is evident from this result that differences in mtHsp70 expression cannot account for the higher rate of protein import in IMF when compared with SS mitochondria. Instead, this difference in basal import rates is likely related to the expression of other import machinery components, as well as differential rates of respiration, as discussed below.

Our main goal was to characterize denervation-induced changes in mitochondrial content in relation to alterations in protein import and organelle respiration. The application of a cell-free in vitro import assay permitted the conclusion that impaired matrix protein import occurs independent of precursor protein availability. Additionally, the import of radiolabeled pOCT into muscle mitochondria, where the background of processed OCT is negligible, provides a useful model to study the import of matrix-destined proteins. Once imported into the matrix, the 39-kDa translation product of OCT is cleaved and processed to the mature 36-kDa form of the enzyme (6). OCT protein import was blunted in both SS and IMF mitochondria from denervated muscle (Fig. 5), and the extent of reduction closely paralleled the denervation-induced decline in whole muscle COX activity (Fig. 2B), which was measured to confirm an overall reduction in muscle oxidative capacity. This is supported by the close correlation between the rate of protein import and whole muscle COX activity (Fig. 8A). Of the two mitochondrial subfractions, import kinetics were reduced to the greatest extent in SS mitochondria, confirming the concept that this subpopulation of mitochondria is more labile during conditions of chronic muscle use and disuse (1, 3, 4, 25). Additionally, differences in import rates between IMF and SS mitochondria from denervated muscle were well matched with alterations in state 3 respiration (Fig. 8B). These data reinforce the contention (38) that protein import is closely related to the rate of ATP formed by respiration, which is used to internally to drive the translocation of the precursor into the matrix.

Our data also suggest the existence of a temporal dissociation between the rate of muscle atrophy and changes in mito-

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Fig. 5. Mitochondrial protein import of OCT in skeletal muscle denervated for 3, 7, and 14 days. A: representative autoradiograms of protein import at 3, 7, and 14 days of denervation. Con, control; Den, denervated; TL, 5 μl of translation product. B: quantification of protein import was expressed as a ratio of denervated/control muscle mitochondria in SS and IMF subfractions (n = 7–8 per group). C: reduced protein import in SS and IMF mitochondria from denervated muscle expressed as a ratio of percentage reduction of OCT import/day of denervation (n = 23). Values are means ± SE.
Mitochondrial content and function during different periods of muscle disuse. For example, after 3 days of denervation, COX activity and state 3 respiration were reduced by 30% and 40%, respectively, but there was only a 9% decline in muscle mass. Later, at 7 and 14 days of denervation, the extent of muscle atrophy was greater than the reduction in oxidative capacity. These data suggest that early adaptations in mitochondrial content and function may be precursors for the initiation of the subsequent cellular activities that are associated with denervation atrophy (9, 30, 31). The reduced mitochondrial content, which is coupled to organelle dysfunction, was demonstrated by a decrease in state 3 respiration (Fig. 3) and an increase in ROS production (Fig. 4) in both SS and IMF mitochondria. An increase in ROS production leads to the activation of proteolytic pathways involving caspasas, the ubiquitin-proteasome system, and autophagy, all of which can promote muscle atrophy (30, 31; Fig. 9). Furthermore, mitochondrial ROS generation is inversely related to mitochondrial respiration (2, 35), and the increase in oxidative stress evident in denervated muscle is likely caused by a combination of reduced antioxidant activity (3), as well as impaired function of complexes within the ETC. These alterations in respiration and ROS production were more pronounced in SS mitochondria, suggesting a greater sensitivity and response to denervation in this mitochondrial subfraction.

Since the protein import machinery mediates the import process, we also determined the expression of selected components within SS and IMF mitochondria from denervated muscle to evaluate their potential roles in disuse-mediated changes in protein import. The content of all components investigated (Tim23, Tom20, and mtHsp70) decreased in SS mitochondria during the time course of denervation. This response was different in IMF mitochondria, where mtHsp70 and Tim23 were significantly reduced at specific time points (Fig. 6). Clearly, components of SS mitochondrial import

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machinery were more vulnerable to denervation-induced organelle remodelling, and these heterogenous adaptations could be related to altered cardiolipin content, since this phospholipid appears to be critical for the initial embedding of the protein components of the inner membrane (20). A greater loss of cardiolipin in SS mitochondria could account for the reduced incorporation of import proteins in SS mitochondria. However, this mechanism requires further investigation.

One important implication of the present study relates to the potential redox modulation of the mitochondrial protein import machinery during oxidative stress. Since large quantities of ROS are responsible for initiating several harmful events within cells, they may inappropriately signal the arrest of precursor protein import. Indeed, the greater ROS production evident in SS mitochondria (2) may serve to attenuate protein precursor protein import. Indeed, the greater ROS production during oxidative stress conditions in skeletal muscle.

In summary, we have shown for the first time that skeletal muscle responds to chronic muscle disuse by reducing the rate of mitochondrial precursor protein import, which is mediated in part by a reduced capacity for ATP production, as well as modifications in the expression of critical components of the import machinery (Fig. 9). Decreased protein import is likely a strong contributor to the reduction in mitochondrial content evident with denervation. In addition, we have provided some evidence for the inhibition of mitochondrial precursor protein import by oxidative stress in muscle mitochondria. This work sets the stage for future studies designed to evaluate the physiological role of the protein import pathway during oxidative stress conditions in skeletal muscle.

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