J Appl Physiol
88: 1601–1606, 2000.

Effect of contractile activity on protein turnover in skeletal muscle mitochondrial subfractions

MICHAEL K. CONNOR, OLGA BEZBORODOVA,
C. PATRICIA ESCOBAR, AND DAVID A. HOOD
Departments of Biology and of Kinesiology and Health Science, York University, Toronto, Canada M3J 1P3

Connor, Michael K., Olga Bezborodova, C. Patricia Escobar, and David A. Hood. Effect of contractile activity on protein turnover in skeletal muscle mitochondrial subfractions. *J Appl Physiol* 88: 1601–1606, 2000.—To determine the role of intramitochondrial protein synthesis (PS) and degradation (PD) in contractile activity-induced mitochondrial biogenesis, we evaluated rates of [^{35}S]methionine incorporation into protein in isolated rat muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. Rates of PS ranged from 47 to 125% greater ($P < 0.05$) in IMF compared with SS mitochondria. Intense, acute in situ contractile activity (10 Hz, 5 min) of fast-twitch gastrocnemius muscle resulted in a 50% decrease in PS ($P < 0.05$) in SS but not IMF mitochondria. Recovery, or continued contractile activity (55 min), reestablished PS in SS mitochondria. In contrast, PS was not affected in either SS or IMF mitochondria after prolonged (60-min) contractile activity in the presence or absence of a recovery period. PD was not influenced by 5 min of contractile activity in the presence or absence of recovery but was reduced after 60 min of contractions followed by recovery. Chronic stimulation (10 Hz, 3 h/day, 14 days) increased muscle cytochrome-*c* oxidase activity by 2.2-fold but reduced PS in IMF mitochondria by 29% ($P < 0.05$; $n = 4$). PS in SS mitochondria and PD in both subfractions were not changed by chronic stimulation. Thus acute contractile activity exerts differential effects on protein turnover in IMF and SS mitochondria, and it appears that intramitochondrial PS does not limit the extent of chronic contractile activity-induced mitochondrial biogenesis.

mitochondrial biogenesis; protein synthesis; protein degradation; chronic stimulation; cytochrome-*c* oxidase

SKELETAL MUSCLE MITOCHONDRIA are found adjacent to the sarcolemma [subsarcolemmal (SS) mitochondria] or between the myofibrils [intermyofibrillar (IMF) mitochondria]. SS mitochondria are thought to provide the energy needed for membrane transport, whereas IMF mitochondria are likely responsible for the generation of ATP for muscle contraction. These mitochondrial subfractions have been shown to possess different biochemical (7, 37), physiological (30), and morphological (29) characteristics. Furthermore, they adapt differently to chronic muscle use and disuse (16, 21, 35). The reason for this may be due, in part, to a differential capacity of each mitochondrial subfraction for protein synthesis. It is well established that mammalian mitochondria possess their own DNA, which encodes 13 polypeptides. Once mitochondrial DNA (mtDNA) is transcribed, the resulting RNA transcripts are translated into proteins, which form an integral part of the respiratory chain complexes and are, therefore, essential for mitochondrial respiration. mtDNA mutations, which give rise to defective or absent protein products, are known to be a main cause of tissue-specific mitochondrial disorders (20). Thus whereas the biogenesis of a functional organelle requires an intact intramitochondrial protein synthesis system, relatively little is known regarding the capacity of mitochondrial protein synthesis to adapt to alterations in tissue functional demand. In skeletal muscle, protein synthesis has been shown to adapt during aging (33) and in response to insulin deficiency (32). One report documented that protein synthesis was elevated in brain and heart mitochondria after swimming exercise (12), but to our knowledge there are no data on the effect of exercise on protein turnover (i.e., synthesis and degradation) in skeletal muscle mitochondrial subfractions. In view of the apparent greater adaptation of SS compared with IMF mitochondria in response to training (16), we hypothesized that protein synthesis in SS mitochondria would be increased more than that in IMF mitochondria as a result of chronic contractile activity. In addition, because both protein synthesis and degradation are energy-dependent processes, we expected that acute, intense contractile activity would reduce these rates in both mitochondrial subfractions but that they would return above control values after a recovery period.

METHODS

Animals and surgery. Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing 300–350 g, were housed individually and were given food and water ad libitum. The procedures involved in the surgical implantation of electrodes for chronic stimulation were as outlined previously (35, 36). Animals were stimulated at 10 Hz (3 h/day) for 14 days. Twenty-one hours after the chronic stimulation period, tibialis anterior (TA) muscles from the stimulated and contralateral nonstimulated limbs were removed, and a small portion was quick-frozen for measurements of cytochrome-*c* oxidase (Cytox) enzyme activity (15). The remainder was used for the isolation of mitochondria (see below).

The surgical preparation, as well as the acute in situ evaluation of muscle performance of the gastrocnemius-plantaris-soleus muscle group, was exactly as described

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previously (13). Muscles were stimulated at 10 Hz via the sciatic nerve for either 5 or 60 min, followed by either no recovery period or a recovery period lasting 55 min.

Mitochondrial isolation. Gastrocnemius (acute stimulation and recovery experiment) or TA (chronic stimulation experiment) muscles were removed, and IMF and SS mitochondrial subfractions were isolated by differential centrifugation as described previously in detail (7, 35, 37). Mitochondria were resuspended in 10 mM HEPES (pH 7.4), 0.25 M sucrose, 2.5 mM potassium phosphate dibasic, 10 mM succinate, 0.21 mM ADP, and 1 mM dithiothreitol, and protein concentrations were measured (2).

Mitochondrial protein synthesis and degradation. SS and IMF mitochondria were diluted to 4 mg/ml. Initiation of protein synthesis was achieved by adding mitochondria (80 μg) to a prewarmed (30°C) medium containing 25 mM MOPS (pH 7.4), 0.1 mM unlabeled amino acids (except methionine), 2 mM ADP, and 20 μM methionine containing 28 μCi of [^{35}S]methionine, as done previously (7, 27). Aliquots (10 μl) were removed at various time points and either subjected to denaturing SDS gel electrophoresis and autoradiography or directly spotted onto Whatman filter paper (3 mm) prewetted with 5% TCA/5 mM methionine. Filter papers were washed four times with 5% TCA/5 mM methionine followed by two washes with ethanol/ether (3:1 vol/vol). Protein synthesis rates, as indicated by [^{35}S]methionine incorporation, were determined by liquid scintillation counting and expressed per milligram of mitochondrial protein. For electrophoresis, mitochondria (50 μg protein) were diluted 20-fold by the addition of buffer containing (in mM) 5 methionine, 100 KCl, 5 $MgSO_4$, 5 EGTA, 1 ATP, and 50 Tris·HCl (pH 7.4). Mitochondria were pelleted and washed twice in the same buffer, and the pellet was dissolved in 2% SDS, 0.1 M Tris·HCl (pH 6.8), 10% glycerol, 2 mM EDTA, and 1% β-mercaptoethanol before being loaded on a 10% polyacrylamide gel. Gels were then subjected to fluorography to visualize the proteins (37).

To evaluate intramitochondrial protein degradation, synthesis reactions were terminated by a 10-fold dilution of the mitochondria with resuspension buffer containing 5 mM unlabeled methionine, as commonly done (1, 9). Mitochondria were then incubated at 30°C, and aliquots were removed at various time points and TCA precipitated, as described above, to measure the decrease in [^{35}S]methionine incorporation over time. Protein degradation rates were determined by liquid scintillation counting.

Statistical analyses. Differences in the rates of both protein synthesis and protein degradation in SS and IMF mitochondria from stimulated and nonstimulated control muscle were evaluated by using two-way ANOVAs. Paired *t*-tests were used to compare Cytox activities in stimulated and contralateral control muscles. Values are presented as means ± SE.

RESULTS

Protein synthesis rates in skeletal muscle SS and IMF mitochondria. Protein synthesis rates in IMF and SS mitochondria were determined by measuring [^{35}S]methionine incorporation into newly synthesized proteins (7, 27). These analyses revealed that synthesis rates in IMF mitochondria were 47% higher ($P < 0.05$) than in SS mitochondria. Absolute rates of [^{35}S]methionine incorporation were 308.2 ± 6.5 and 209.1 ± 13.5 disintegrations·min^{-1}·mg^{-1}, respectively (Fig. 1*A*). The greater rate of protein synthesis observed in IMF mitochondria is consistent with previous results from our laboratory (7). In addition, this higher rate of amino acid incorporation does not appear to be confined to any specific protein, as radioactivity present in each of the 13 mitochondrially encoded proteins is higher in IMF compared with SS mitochondria (Fig. 1*B*). Thus IMF mitochondria possess a greater capacity for protein synthesis under nonadaptive, steady-state conditions.

Effect of acute contractile activity and recovery on protein turnover in SS and IMF mitochondria. To evaluate the effects of acute contractile activity and recovery on intramitochondrial protein turnover, SS and IMF mitochondria were isolated either immediately after (zero recovery) or 55 min subsequent to the cessation of in situ contractile activity induced by 10-Hz electrical stimulation for either 5 or 60 min. [^{35}S]methionine incorporation rates in SS mitochondria were reduced ($P < 0.05$) by 51.6 ± 18.9% after only 5 min of contractile activity but returned to control levels after 55 min of recovery (Fig. 2*A*). In contrast, IMF mitochondria were unaffected by 5 min of contractile activity, but amino acid incorporation into protein was reduced by 39.5 ± 5.2% ($P < 0.05$) after the combina-

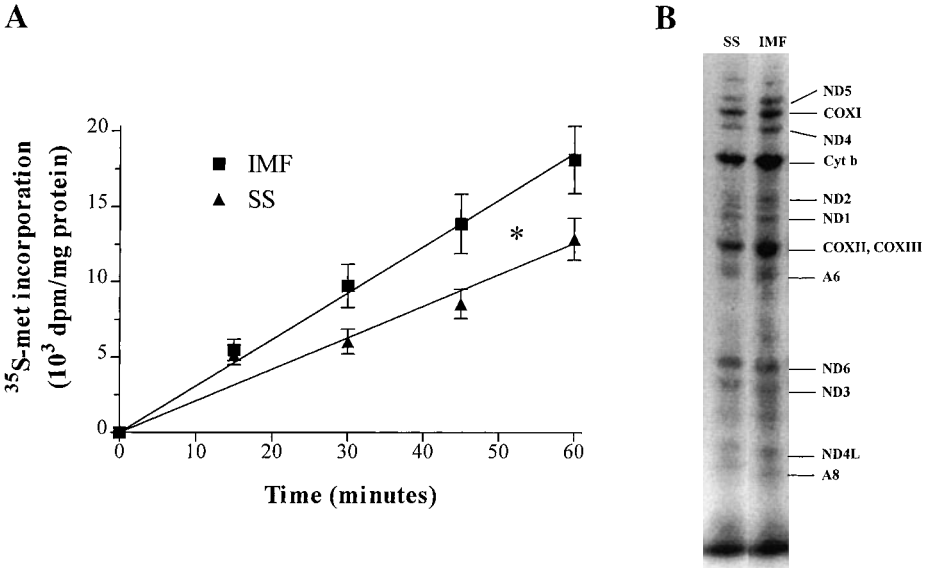

Fig. 1. *A*: sarcolemmal (SS) and intermyofibrillar (IMF) mitochondria were isolated from rat gastrocnemius muscle (n = 24 animals) and were incubated at 30°C in presence of [^{35}S]methionine (met). Protein synthesis rates were determined by measuring [^{35}S]methionine incorporation by liquid scintillation counting. *$P < 0.05$ compared with nonstimulated muscle. *B*: autoradiogram identifying the 13 mitochondrially encoded peptides produced from a typical protein synthesis reaction in SS and IMF mitochondria (tentatively identified based on Ref. 5). Equal amounts of mitochondrial protein (50 μg) were loaded in each lane. ND1, 2, 3, 4, 4L, 5, and 6: NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6; COXI, II, and III: cytochrome-*c* oxidase subunits I, II, and III; Cyt b: cytochrome-*b*; A6 and A8: H^+-ATPase subunits 6 and 8.

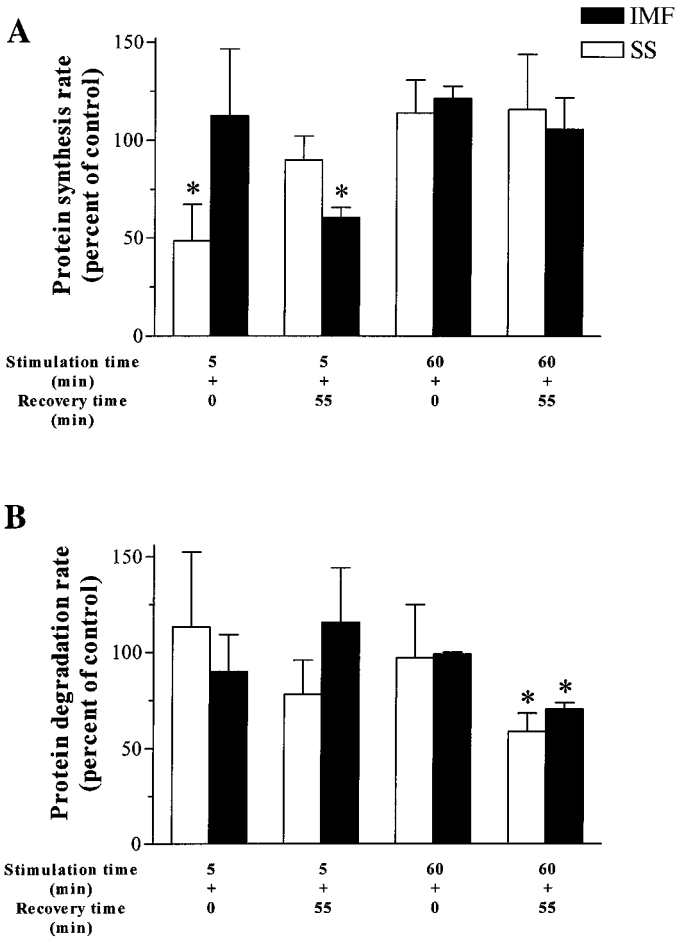

Fig. 2. *A*: protein synthesis rates in isolated IMF and SS skeletal muscle mitochondria in response to in situ electrical stimulation (10 Hz) of gastrocnemius-plantaris-soleus muscle group for either 5 or 60 min with or without 55 min of recovery (n = 4–8 animals). SS and IMF mitochondria were then isolated immediately, and protein synthesis rates were determined by measuring [^{35}S]methionine incorporation as in Fig. 1. Values are expressed as a percentage of those found in mitochondria from nonstimulated control muscle. *B*: protein degradation rates in isolated SS and IMF skeletal muscle mitochondria (n = 3–6 animals). After contractile activity with or without recovery as in *A*, mitochondrial protein synthesis was allowed to proceed for 60 min. Synthesis reactions were then stopped (*time 0*), and mitochondria were incubated at 30°C for various time intervals between 0 and 20 min. Protein degradation rates were determined by measuring slope of the decline in [^{35}S]methionine incorporation relative to the value at *time 0*. These slopes were then expressed as a percentage of those found in mitochondria from nonstimulated control muscle. *$P < 0.05$ compared with nonstimulated muscle.

tion of 5 min of contractile activity and 55 min of recovery (Fig. 2*A*). After 60 min of contractile activity either in the presence or absence of a recovery period, [^{35}S]methionine incorporation in the IMF and SS subfractions was not different from that evident in mitochondria isolated from contralateral nonstimulated control muscle (Fig. 2*A*).

Unlike protein synthesis, there was no apparent influence of 5 min of contractile activity on mitochondrial protein degradation, either in the presence or absence of a recovery period (Fig. 2*B*). In addition, 60 min of contractile activity elicited no alteration in protein degradation rate in either mitochondrial subfractions (Fig. 2*B*). However, after recovery from 60 min of stimulation, there were marked 41.0 ± 9.3 and 29.7 ± 3.5% decreases in the rates of protein degradation in SS and IMF mitochondria, respectively. This suggests the occurrence of a protein-stabilizing influence of contractile activity that is manifest only during the recovery period.

Effects of chronic contractile activity on mitochondrial protein synthesis and degradation. To evaluate the effects of long-term elevations in contractile activity on protein synthesis and degradation, rat TA muscles were electrically stimulated (10 Hz, 3 h/day) for 14 days. This treatment effectively induced a 2.2 ± 0.3-fold increase ($P < 0.05$) in Cytox activity (Fig. 3*A*), an enzyme that contains three essential subunits derived from the mitochondrial genome. When expressed relative to the specific activity of the incubation medium, protein synthesis rates in control IMF and SS mitochondria were 115 and 51 pmol methionine·mg protein^{-1}·h^{-1}, respectively. These rates are in the range reported by others (1, 27). Chronic stimulation induced a 29% reduction ($P < 0.05$) in the rate of [^{35}S]methionine incorporation in IMF mitochondria compared with IMF mitochondria from nonstimulated TA (Fig. 3*B*). In contrast, chronic stimulation elicited no change in the [^{35}S]methionine incorporation in SS mitochondria (Fig. 3*C*). Intramitochondrial protein degradation also remained unaffected by chronic stimulation, as evident from the similar rates of decline in [^{35}S]methionine incorporation in SS and IMF mitochondria in both stimulated and nonstimulated control muscle (Fig. 3*D*). Thus these data indicate that 14 days of contractile activity suppressed protein synthesis in IMF but not SS mitochondria and that this response did not appear to inhibit the increase in Cytox enzyme activity brought about by the treatment.

DISCUSSION

mtDNA contains 13 genes, the products of which form an integral part of the respiratory chain. mRNAs transcribed from mtDNA are translated into proteins within the organelle and then inserted into the mitochondrial inner membrane. In this study, we evaluated protein synthesis in two mitochondrial subfractions obtained from skeletal muscle and investigated whether rates of mitochondrial protein turnover can be altered by either acute or chronic contractile activity.

Previous investigations of mitochondrial protein synthesis have established that a high cellular energy state is required for optimal amino acid incorporation into protein (18, 26). A prime motive for the use of our contractile activity model was the recognition that an alteration in cellular energy state can occur in skeletal muscle subject to intense 10-Hz contractile activity and that it is readily and rapidly reversible during the recovery period (13). Thus we chose to investigate both a short (5 min) and a longer period (60 min) of acute 10-Hz contractile activity, anticipating that 5 min was of sufficient duration to induce profound muscle fatigue to ~35% of initial tension, as well as a severe metabolic

stress (i.e., decreased pH, ATP, phosphatidylcholine, and glycogen levels and elevated lactate concentrations; Ref. 13), but that a longer time (i.e., 60 min) might be necessary for the alteration in metabolism to have an effect on rates of protein turnover. In addition, it is now recognized that events occurring during recovery from acute exercise are important for the subsequent manifestation of activity-induced adaptations over a longer term as a result of training (11, 28, 38). Our results indicate that only 5 min of intense contractile activity can markedly reduce [^{35}S]methionine incorporation in SS mitochondria and that this is restored after either a recovery period or continued contractile activity for up to 1 h. Whereas this may appear paradoxical, it is known that, during this continued contractile activity, the metabolic changes that occur initially can be largely reversed. For example, ATP, phosphatidylcholine, and lactate levels are almost completely restored to resting values in mixed fast-twitch muscle samples, despite continuous 10-Hz contractile activity for 60 min (13). Thus a metabolic disturbance and its restoration may represent a reasonable explanation for the change in protein synthesis observed in SS mitochondria. However, this could not explain the [^{35}S]methionine incorporation response in the IMF mitochondrial subfraction, because it remained unaffected by 5 min of contractile activity but displayed a reduced synthesis rate after the recovery period. This suggests that factors other than alterations in metabolism play a role in regulating rates of protein synthesis within IMF mitochondria. One explanation may be related to the accumulation of intramitochondrial calcium levels, a pathway that is now viewed as having considerable significance in the control of enzyme activity (25), intracellular signaling, and apoptosis (17). It is known that mitochondrial protein synthesis is highly dependent on the presence of calcium (18) and that calcium uptake into skeletal muscle mitochondria varies between fiber types (34) and between mitochondrial subfractions (30). Palmer et al. (30) have shown that heart IMF mitochondria can accumulate up to 50% more calcium than can SS mitochondria. Furthermore, a mitochondrial matrix calcium-binding protein has been identified (i.e., calmitine; Ref. 23), but the expression of this protein in different mitochondrial fractions has yet to be described. Thus the role of mitochondrial calcium uptake during contractions and recovery in the context of regulating intramitochondrial protein turnover remains as a testable possibility to explain the differences observed in the present study. Regardless, our data indicate that protein synthesis in SS and IMF mitochon-

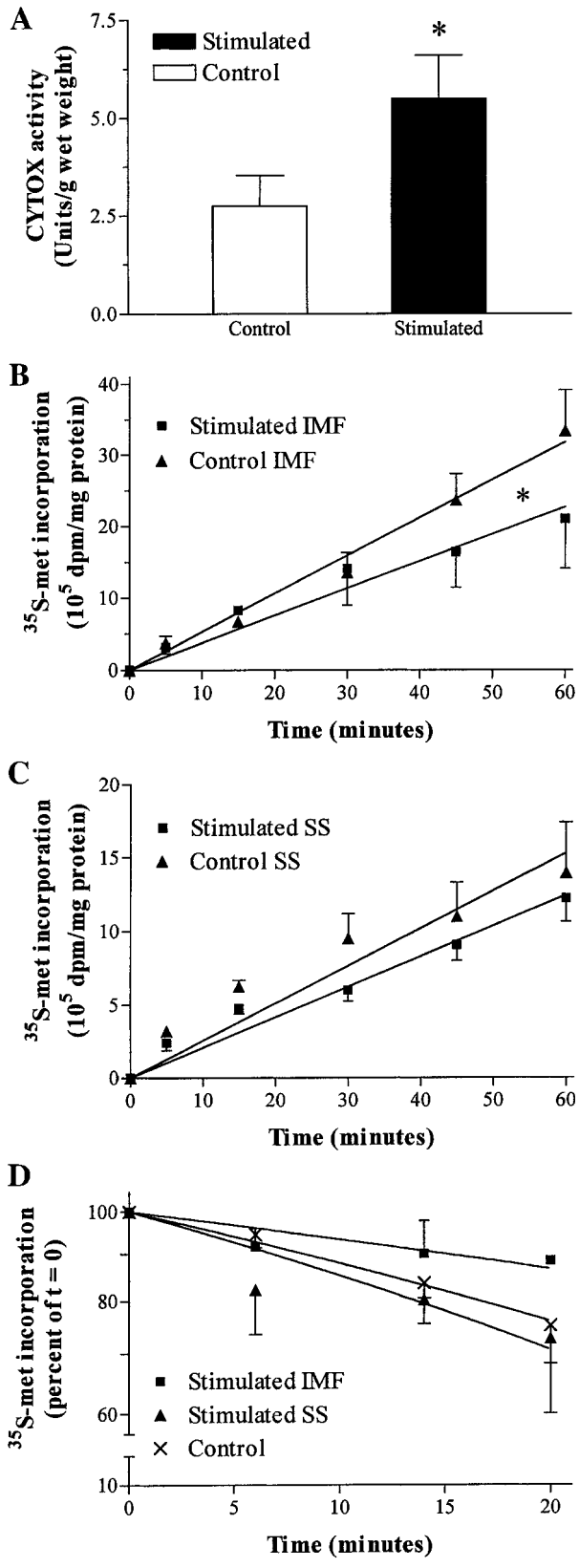

Fig. 3. Rat tibialis anterior (TA) muscles were electrically stimulated (10 Hz, 3 h/day) for 14 days (n = 4 animals). *A*: cytochrome-*c* oxidase (Cytox) activity in extracts from chronically stimulated and nonstimulated control TA muscle. *B*: intact IMF mitochondria were isolated from chronically stimulated and control TA and incubated with [^{35}S]methionine. Synthesis rates were determined by measuring [^{35}S]methionine incorporation into proteins and are expressed per milligram of mitochondrial protein. *C*: protein synthesis in SS mitochondria from chronically stimulated and nonstimulated TA as in *B*. *D*: protein degradation in SS and IMF mitochondria from chronically stimulated and control skeletal muscle. Because degradation rates in control SS and IMF mitochondria were not significantly different, they were combined for clarity. *t*, Time. * $P < 0.05$ compared with nonstimulated muscle.

drial subfractions is differentially sensitive to contractile activity and recovery.

In contrast to the alterations in protein synthesis observed, rates of protein degradation remained unaffected by 5 min of contractile activity in the presence or absence of a recovery period. However, when 60 min of contractile activity were followed by a recovery period, a significant reduction in degradation rate was observed in both mitochondrial subfractions. This suggests an important mechanism for the accumulation of protein within the organelle, as muscle adapts to intermittent bouts of acute contractile activity, and it may help explain the more rapid increase in mitochondrial protein levels when intermittent (11) vs. continuous stimulation (42) is used. The data also indicate that mitochondrial protein synthesis and degradation are regulated differently by contractile activity and that the SS and IMF mitochondrial subfractions do not appear to differ with respect to the regulation of intramitochondrial proteolysis.

Our data obtained in isolated mitochondria do not, in general, resemble the pattern observed for mixed muscle protein turnover. In response to acute exercise, muscle protein synthesis appears to exhibit an immediate decrease (14). This is similar to our finding in SS but not IMF mitochondria. A few hours later, mixed muscle protein synthesis is elevated above preexercise conditions, and it remains elevated for 24 h (4, 31). Perhaps our results would have resembled those of mixed muscle had we chosen a more prolonged recovery period for evaluation. Rates of mixed muscle protein degradation are either unaffected (10) or increased (19) for a prolonged period (31) by contractile activity, whereas our data indicate a reduction in SS and IMF mitochondrial protein degradation during the recovery period after a 60-min period of contractile activity. Thus these differences can be attributed either to the variations in exercise protocols employed or, more interestingly, to the possibility that mitochondrial protein turnover is regulated differently in response to contractile activity compared with the turnover of mixed muscle proteins.

Previous work has shown that the mitochondrial adaptation to 10-Hz contractile activity includes both transcriptional and posttranscriptional events (11, 15, 40). Specifically, we have shown that the increase in Cytox activity observed after 14 days of chronic stimulation exceeded the elevation in Cytox subunit mRNAs, suggesting the possibility that posttranslational events could have influenced the activity-induced increases in enzyme activity at that time (15). Thus we chose to evaluate intramitochondrial protein synthesis and degradation after 14 days of chronic stimulation. Surprisingly, the 2.2-fold increase in Cytox activity observed was not accompanied by increases in protein synthesis or decreases in protein degradation. Indeed, rates of protein synthesis were almost 30% lower in IMF mitochondria isolated from chronically stimulated muscle. The result is also surprising in view of the fact that the largest adaptation in response to chronic contractile activity occurs with the SS mitochondria (16). We hypothesized that, despite the lower inherent rates of protein synthesis in this subfraction compared with IMF mitochondria, SS mitochondrial protein synthesis would show a dramatic reversal in response to chronic contractile activity. Such was not the case, as rates of protein synthesis in SS mitochondria were not affected by the treatment. These data suggest that increases in intramitochondrial protein synthesis are not vital for contractile activity-induced increases in mitochondrial biogenesis. Furthermore, they imply that intramitochondrial translation rates are limited by upstream processes such as mtDNA replication and the availability of mRNA via mtDNA transcription during the adaptation to chronic contractile activity. A similar lack of increase in mitochondrial protein synthesis was reported in liver mitochondria undergoing organelle biogenesis (3). However, thyroid hormone-induced mitochondrial biogenesis in heart was accompanied by an increase in rates of mitochondrial translation (8). Thus the induction of mitochondrial protein synthesis is clearly both tissue and stimulus specific, and contractile activity in skeletal muscle as used in the present study does not invoke any increases in this process. However, it is known that experimentally induced reductions in mitochondrial protein synthesis, of a greater magnitude than observed in this study, result in large decreases in Cytox activity (24) and inhibit the contractile activity-induced increase in Cytox activity normally observed (41). This suggests that the reduction in mitochondrial protein synthesis that we found did not reach a critical level that would impair the increase in tissue Cytox noted in this study. It is interesting that the decrease in protein synthesis observed in IMF mitochondria following chronic stimulation may invoke changes in nuclear gene expression, because it is known that inhibition of intramitochondrial translation can produce a stabilization of nuclear-encoded mRNAs (6). This may form part of the reason for the early increase in cytochrome-*c* mRNA stability that we have observed in response to contractile activity (11). Additionally, it may be involved in the mitochondrial-to-nuclear signaling, which is responsible for the upregulation of nuclear genes under conditions of mtDNA depletion (22, 39). These intriguing possibilities remain to be tested.

Address for reprint requests and other correspondence: D. A. Hood, Dept. of Biology, York Univ., Toronto, Ontario, Canada M3J 1P3 (E-mail: dhood@yorku.ca).

Received 10 December 1999; accepted in final form 24 January 2000.

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