Response of mitochondrial enzymes to decreased muscular activity

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Rifffenberick, David H., James G. Gamble, and Stephen R. Max. Response of mitochondrial enzymes to decreased muscular activity. Am. J. Physiol. 225(6): 1295-1299. 1973. Previous studies have shown that mitochondria isolated from atrophic rat gastrocnemius muscles are deficient in respiratory control. The present study was designed to inquire into the nature of the deficiency. Disuse atrophy was produced by immobilization of the hindlimb by surgical pinning. At various times after pinning, mitochondria were isolated from atrophic and contralateral control muscles. The yield of mitochondrial protein decreased throughout the course of atrophy. Concurrent decrease in the cytochrome oxidase and monoamine oxidase activities per total muscle homogenate suggested that there were fewer mitochondria in atrophic than in control muscles. The specific activities of monoamine oxidase and cytochrome oxidase in isolated mitochondria were unchanged, while the specific activity of malate dehydrogenase was diminished on the 1st day and decreased to 35% of control by the 15th day after immobilization. The loss of malate dehydrogenase activity was probably not a reflection of permeability changes in the mitochondrial membranes since nicotinamide nucleotide, the Ca2+ levels, and NADH oxidation were not different from controls. The loss of a matrix enzyme with sparing of inner and outer membrane enzymes may be a reflection of different rates of turnover of these mitochondrial components. The data support our hypothesis that a mitochondrial deficit is important in the progression of muscular atrophy.

Muscular atrophy; muscle mitochondria; disuse; skeletal muscle

The biochemical and physiological properties of skeletal muscle respond to numerous adaptive and stressful conditions (7). One such condition is inactivity, which causes marked atrophy, of which the biochemical basis is incompletely understood. A recent report from this laboratory showed that mitochondria isolated from atrophic rat gastrocnemius muscles are deficient in respiratory control (20). By the 6th day following the production of disuse, respiratory control was absent, using glutamate as substrate. The loss of respiratory control was accompanied by decreased ADP:O ratios. Studies of the rates of substrate utilization will be presented in a forthcoming paper (unpublished observations). In addition, mitochondria in atrophic muscles are morphologically damaged (32). It was suggested that mitochondrial defects may be of importance in the progression of atrophy. In the present studies on the mitochondrial response to disuse, we have measured the activities of three enzymes: monoamine oxidase (EC 1.4.3.4), cytochrome oxidase (EC 1.9.3.1), and malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37). These enzymes are markers for the outer membrane, the inner membrane, and the mitochondrial matrix, respectively (27, 28). The results of these measurements suggest that atrophic muscles contain fewer mitochondria than control muscles and that the remaining mitochondria display normal activity of monoamine oxidase and cytochrome oxidase but show a pronounced loss of malate dehydrogenase activity. These data support the hypothesis that mitochondria do not necessarily turn over as a unit but that individual components may turn over separately (2, 9, 24). Furthermore, together with studies showing enhanced mitochondrial activity in overuse (16, 17, 24), they suggest a biochemical basis for activity-related alterations in the size and properties of skeletal muscle fibers. These results have been presented in recent preliminary reports (23, 26).

MATERIALS AND METHODS

The materials used and their sources were heparin (grade I, sodium salt), sodium malate, cytochrome c (mammalian, type II), NAD+, sodium ascorbate, sodium oxalacetate, and NADH (Sigma); N,N',N',N'-tetramethyl-p-phenylenediamine dichloride, Triton X-100, and tolenue (Eastman); calcium standard and lanthanum chloride (Harleco); and [2-14C]tryptamine bisuccinate, New England Nuclear. Tissue homogenizers (Tenbroeck) were a product of the Kontes Glass Company. Lubrol-WX was obtained from ICI International. (No longer available from this source, but currently available from Sigma.)

Adult, male, albino rats of the Wistar strain, weighing 250-350 g, were used in all experiments. They were purchased from the Charles River Breeding Laboratories, Inc., and were fed Purina rat chow ad libitum. Disuse atrophy was produced by skeletal fixation, as described by Solandt et al. (30) and modified by Fischbach and Robbins (11). In this procedure, a 25-gauge needle is driven through the calcaneous into the shaft of the distal tibia and a 21-gauge needle is driven through the distal femur into the proximal tibia. In this manner, the limb is immobilized and disuse atrophy is the result. The progression of muscle weight loss after limb immobilization, as well as muscle histology, has been described in a previous publication (22). The contralateral gastrocnemius muscle served as control in all experiments. Comparison of contralateral muscles with gastrocnemius muscles from normal, unoperated animals has shown them to be identical in all respects.

1295
Mitochondria were prepared from homogenates of rat gastrocnemius muscles as described elsewhere (21). The yield of mitochondria from control muscles was about 3 mg mitochondrial protein per gram fresh weight of muscle (21).

Malate dehydrogenase was activated with Triton X-100 and its activity was measured by following the rate of oxidation of NADH in the presence of oxalacetate according to the method of Dupourque and Kun (10). KCN was included in the assay medium (27). The decrease in absorbance at 340 nm was determined with a Gilford recording spectrophotometer. To obviate the possibility that changes in malate dehydrogenase activity in mitochondria from atrophic muscles might be caused by increased fragility and sensitivity to Triton X-100, Lubrol-WX (28) was employed as an “activator” in some experiments. The results obtained with Lubrol-WX and Triton X-100 were identical. In a few experiments, malate dehydrogenase activity was measured in the opposite direction, viz., utilizing malate and NAD+ and following the increase in absorbance at 340 nm.

Cytochrome oxidase was measured polarographically, as described by Schreiber et al. (29). The enzyme was activated with Lubrol-WX according to Schnaitman and Greenawalt (28).

Monoamine oxidase was measured radiometrically using [2-14C]tryptamine bisuccinate (sp act 2.23 X 10^3 dpm/nmole) as substrate, according to Wurtman and Axelrod (33). The assay was carried out at pH 7.9, which we determined to be optimum for monoamine oxidase in skeletal muscle. Counting of radioactivity was done in a Packard scintillation spectrometer at 70% efficiency.

NAD specific isocitrate dehydrogenase was assayed according to Schnaitman and Greenawalt (28).

Protein concentrations were determined by the method of Lowry et al. (18), using crystalline bovine serum albumin (Sigma) as the standard.

Calcium was determined by the method of Carafoli and Lehninger (3) using a Perkin-Elmer atomic-absorption spectrophotometer.

Total nicotinamide nucleotides were determined by the methyl ethyl ketone method (5, 6) after conversion of reduced nicotinamide nucleotides in the mitochondria to the oxidized form (25).

RESULTS

The yield of mitochondrial protein decreased progressively throughout atrophy and was markedly reduced (−40%) by day 7 (Fig. 1). The total homogenate activity of monoamine oxidase and of cytochrome oxidase, taken as markers for the outer and inner membranes, respectively, were measured to determine whether the diminished yield of mitochondrial protein depicted in Fig. 1 resulted from a decreased number of mitochondria in atrophic muscle or from altered sedimentation characteristics of damaged mitochondria. Figure 2 shows that the activity of monoamine oxidase per muscle decreased to about 70% of the control value by the 9th day after skeletal fixation, and Fig. 3 shows a similar decrease in the activity per muscle of cytochrome oxidase. These results suggest that atrophic fibers contain fewer mitochondria than control muscles.

The activities of mitochondrial enzyme markers for outer and inner membranes and the matrix space were determined. Representative values for the specific activities in control mitochondria of monoamine oxidase, cytochrome oxidase, and malate dehydrogenase are 0.49, 2,140, and 4,950 nmoles/min per mg mitochondrial protein, respectively. The specific activity of malate dehydrogenase in isolated mitochondria decreased markedly by the 1st day after immobilization and reached a value of 35% of control by day 15 (Fig. 4). The specific activities of monoamine oxidase (Fig. 5) and cytochrome oxidase (Fig. 6) in iso-
MITOCHONDRIAL ENZYMES IN MUSCULAR ATROPHY

FIG. 4. Effect of limb immobilization on malate dehydrogenase activity in isolated mitochondria. Data, determined as nmoles NADH oxidized/mg mitochondrial protein per min, are presented as % of control, average ± SD of 4 determinations. Experimental procedures as described in text.

FIG. 5. Effect of limb immobilization on monoamine oxidase activity in isolated mitochondria. Data, determined as nmoles product formed/mg mitochondrial protein/mm, are presented as % of control, average ± SD of at least 4 determinations. Experimental procedures as described in text.

FIG. 6. Effect of limb immobilization on cytochrome oxidase activity in isolated mitochondria. Data, determined as nmoles O2 consumed/mg mitochondrial protein/min, are presented as % of control, average ± SD of at least 4 determinations. Experimental procedures as described in text.

Three mitochondria were unchanged at all stages in the course of disuse atrophy.

The Km of malate dehydrogenase with respect to malate in mitochondrial preparations from atrophic and control muscles was identical (1.2 mM). Furthermore, addition of mitochondria from atrophic muscle caused no inhibition of malate dehydrogenase in control preparations (unpublished observations).

The specific activity of the mitochondrial NAD-specific isocitrate dehydrogenase was also decreased to 60% of the control value by the 10th day following limb immobilization (unpublished observations).

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To assess the possibility that loss of matrix enzymes was simply due to permeability changes of the mitochondrial membrane systems, the levels of calcium and of total nicotinamide nucleotides were determined. The content of mitochondria of both of these materials was the same in preparations from atrophic or control muscles (Tables 1 and 2). In addition, mitochondria from atrophic muscles did not show increased oxidation of added NADH (unpublished observation).

DISCUSSION

The data presented in this paper are in agreement with our previous observation that mitochondria isolated from disused rat gastrocnemius muscles are deficient in functional activity (20).

Figure 1 shows that the amount of mitochondrial protein recovered from atrophic muscles is less than that obtained from controls. The decreased yield would reflect two possibilities: a) that there are actually fewer mitochondria in the atrophic myofiber, or b) that mitochondria in atrophic muscles are fragile and were disrupted during homogenization so that their sedimentation characteristics were altered. The observed decrease in the total activity per muscle of monoamine oxidase (Fig. 2) and of cytochrome oxidase (Fig. 3) suggests that there are, in fact, fewer mitochondria in atrophic muscles than in controls, since total homogenate activities of these enzymes would not be affected by changes in sedimentation of mitochondria.

It is of interest that while the total activity of cytochrome oxidase and monoamine oxidase per muscle decreased throughout the course of atrophy, the specific activities of these enzymes in isolated mitochondria were the same in disused and control preparations (Figs. 5 and 6). Thus, the inner and outer membranes may be structurally intact. On the other hand, the specific activity of malate dehydrogenase, a matrix enzyme (28), decreased markedly after limb immobilization to 35% of control by day 15 (Fig. 4). The cause of the selective loss of a matrix enzyme with sparing of inner and outer membrane enzymes is not

<table>
<thead>
<tr>
<th>Days After Skeletal Fixation</th>
<th>No. of Rats</th>
<th>Atrophic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>78.08 ± 41.42</td>
<td>60.95 ± 6.04</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>71.95 ± 34.90</td>
<td>60.91 ± 10.36</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>44.11 ± 3.90</td>
<td>56.26 ± 14.98</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>43.11 ± 11.33</td>
<td>47.79 ± 9.81</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>68.80 ± 15.45</td>
<td>81.21 ± 36.71</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>76.47 ± 11.03</td>
<td>67.34 ± 9.91</td>
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</table>

Experimental conditions as described in the text. All values are averages ± SD.

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<thead>
<tr>
<th>Days After Skeletal Fixation</th>
<th>No. of Rats</th>
<th>Atrophic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>13.98 ± 6.37</td>
<td>10.29 ± 1.08</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>14.27 ± 2.46</td>
<td>16.32 ± 1.28</td>
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<tr>
<td>6</td>
<td>15.29 ± 3.47</td>
<td>12.03 ± 3.99</td>
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Total nicotinamide nucleotides were determined as described in the text. Numbers are averages ± SD of at least 4 determinations.
apparent. The diminished activity of malate dehydrogenase is not attributable to alteration of enzyme protein since the $K_m$ of the enzyme with respect to malate in mitochondrial preparations from atrophic and control muscles was identical (1.2 mM, data not shown). Furthermore, a mixing experiment showed no inhibition by mitochondria from atrophic muscle of malate dehydrogenase activity in the control preparation (data not shown). Thus, the decreased activity of malate dehydrogenase is probably the result of diminished amounts of enzyme. The loss of enzyme is probably not attributable simply to leakage from the mitochondria since the levels of calcium (Table 1) and of total nicotinamide nucleotides (Table 2) were not changed. The levels of these mitochondrial components might be expected to be altered by permeability changes of the mitochondria, although it is possible that the calcium data may reflect accumulation rather than leakiness of the mitochondria. Selective permeability of the mitochondria to enzymes cannot be ruled out. To evaluate this possibility, it would be of obvious interest to compare total homogenate malate dehydrogenase activities in atrophic and control preparations to assess changes which might result from leakage of the enzyme from mitochondria. Unfortunately, mitochondrial activity is only 2-3% of the total activity (18). Thus, even total leakage from mitochondria would not be detectable. Studies of cytosol and mitochondrial isoenzyme distribution would be helpful and will be the subject of future work.

It seems possible that the selective loss of malate dehydrogenase represents a facet of mitochondrial turnover. It is now apparent that mitochondria do not turn over as a unit as originally suggested by the work of Fletcher and Sanadi (12), but that some mitochondrial components may turn over separately (2, 9). In fact, Aschenbrenner et al. (1) have concluded that $\delta$-aminolevulinate synthetase “and probably other matrix enzymes, can be degraded independently of the mitochondrial membrane systems.” In this regard, the interesting experiments of Holloszy and coworkers (16, 17, 24) appear to be related to the present study. Holloszy demonstrated increased mitochondrial activity in response to overuse of rat skeletal muscles. Increases in mitochondrial components were selective in that the activities of certain enzymes increased, whereas others were unchanged. The work of Holloszy, together with the present investigation, shows that skeletal muscle mitochondria are “plastic,” in that they can change in the number and in activity in response to increased or decreased energy demand of the muscle cell.

The mitochondrial alterations described in this paper are similar to the findings of Carafoli et al. (4), who demonstrated changes in mitochondria isolated from denervated pectoralis muscles of the pigeon. There are several important differences between the results of Carafoli et al. (4) and the present study. Diminution of the number of mitochondria in denervated pigeon breast muscles was accompanied by loss of both membrane and matrix components, in contrast to the more selective loss in response to immobilization described in this report. This discrepancy may possibly be accounted for by differences in the experimental models employed in these studies. Denervation causes more than simple disuse and results in fibrillations, changes in acetylcholine sensitivity, nuclear proliferation, and a host of other alterations (13, 15). Skeletal fixation is considered to be the better model for production of simple disuse since it is not complicated by the rather drastic effects of nerve section (14). Furthermore, Carafoli et al. (4), employing the Warburg apparatus, reported no change in respiratory control, whereas our studies with the oxygen electrode (20) clearly demonstrated such changes. In addition, Margreth et al. (19) have reported that malate dehydrogenase activity is diminished in mitochondria from denervated muscle.

Our results are also similar in some respects to those of studies of effects of exhaustive exercise by Dohm et al. (8), who reported decreased mitochondrial yield, respiratory control, and oxygen utilization, with unaltered ADP:O ratios. Terjung et al. (31), on the other hand, found no changes in these parameters. The nature of possible factors which might cause mitochondrial dysfunction in such disparate situations as disuse and exhaustive exercise is not known.

Whether the mitochondrial alterations described above are important in the primary stages of muscular atrophy will not be known until the cause of atrophy is identified. It is possible that mitochondrial damage may be caused by lysosomal enzyme activities previously shown to be enhanced in disuse atrophy (22), but the present state of our knowledge of the temporal sequence of events in atrophy does not permit a clear-cut conclusion to be drawn. Nevertheless, since the loss of respiratory control (20) and of malate dehydrogenase activity (Fig. 6) appears almost immediately after skeletal immobilization, mitochondrial deficiencies may be of significance in the initial stages of atrophy.

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MITOCHONDRIAL ENZYMES IN MUSCULAR ATROPHY


