Cytochrome c mRNA in skeletal muscles of immobilized limbs

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Booth, Frank W., Wei Lou, Marc T. Hamilton, and Zhen Yan. Cytochrome c mRNA in skeletal muscles of immobilized limbs. J. Appl. Physiol. 81(5): 1941–1945, 1996.—Even though immobilization of a slow skeletal muscle in a lengthened position prevents muscle atrophy, it is unknown whether this treatment would prevent a decrease in mitochondrial quantity. We found that, regardless of muscle length in immobilized limbs, the mRNA of a marker for mitochondrial quantity, cytochrome c, decreased. Cytochrome c mRNA per milligram of muscle was 62 and 72% less 1 wk after fixation of the soleus muscle in shortened and lengthened positions, respectively, than age-matched controls. Cytochrome c mRNA per milligram of muscle was 36 and 32% less in the tibialis anterior muscle fixed for 1 wk in the shortened and lengthened positions, respectively, compared with age-matched controls. Recently, in the 3'-untranslated region of cytochrome c mRNA a novel RNA-protein interaction that decreases in chronically stimulated rat skeletal muscle was identified (Z. Yan, S. Salmons, Y. L. Dang, M. T. Hamilton, and F. W. Booth, Am. J. Physiol. 271 (Cell Physiol. 40): C1157–C1166, 1996). The RNA-protein interaction in the 3'-untranslated region of cytochrome c mRNA in soleus and tibialis anterior muscles was unaffected by fixation in either shortened or lengthened position. We conclude that, whereas lengthening muscle during limb fixation abates the loss of total muscle protein, the percentage decrease in cytochrome c mRNA is proportionally greater than total protein. This suggests that the design of countermeasures to muscle atrophy should include different exercises to maintain total protein and mitochondria.

MATERIALS AND METHODS

Animals. Pathogen-free Sprague-Dawley female rats were obtained from Harlan (Houston, TX) and assigned randomly at 7–8 wk of age to control group and hindlimb immobilization groups with ankles fixed in either plantar flexion or dorsiflexion. Rats were anesthetized with 1.4 ml/kg of a mixture of ketamine (54 mg/ml), xylazine (2.2 mg/ml), and acepromazine (3.5 mg/ml) during the application of immobili-
zation to both hindlimbs with plaster of Paris (5) and at the time of death.

Total protein. Total protein was determined with a detergent-compatible protein assay (Bio-Rad).

RNA isolation. RNA for Northern blot analysis was extracted from soleus and tibialis anterior (TA) muscles of three groups of rats: ankles fixed for 7 days in planar flexion, ankles fixed for 7 days in dorsiflexion, or controls who were killed at the end of immobilization according to a procedure by Chomczynski and Sacchi (7) with RNAzol B (Biotex Laboratories) as described earlier (32).

Northern blot analysis. The coding region of the cytochrome c gene was subcloned into pBluescript SK (+) vector (Stratagene). In brief, the BamHI fragment (958-base pair) from pRC4 plasmid (25) was inserted into the polylinker region of pBluescript SK (+) digested with BamHI and EcoR I. The resulting plasmid pRC4 (Bluescript) was used for Northern blot analysis of cytochrome c mRNA as described by Babij and Booth (2). mRNA was quantified by image analysis of the autoradiogram (BioImage, Millipore). Total RNA per milligram wet weight was determined by the alkaline hydrolysis procedure of Munro and Fleck (20).

Cytoplasmic extract. Muscle homogenization was described in Yan et al. (32). The homogenates were centrifuged at 15,000 g for 15 min at 4°C. The supernatant (S15) was snap-frozen in liquid nitrogen and stored at −80°C until analysis. The protein concentrations of S15 were determined by the Lowry method (18).

Radiolabeled-RNA probe. For the cytochrome c 3′-UTR probe, pRC4CAT3′ (9) was digested with Bgl II and BamHI followed by ligation with T4 ligase into pBluescript SK (+). A 116-base pair fragment (+1337 to +1452) was produced by digestion with Dra I and in vitro transcription with T3 polymerase (Promega) and [α-32P]Juridine 5′-triphosphate (3,000 Ci/mmol; ICN). After transcription at 37°C for 1 h, RNA transcripts were digested with 1 unit of ribonuclease-free deoxyribonuclease (Promega) for 15 min at 37°C, extracted with phenol-chloroform, and precipitated in ethanol with 20 µg Escherichia coli RNA as carrier. The radiolabeled RNAs were quantified, and their specific activities (counts·min⁻¹·µg⁻¹) were determined by liquid scintillation counting after trichloroacetic acid precipitation (24).

Ultraviolet (UV) cross-linking. UV cross-linking assays were performed according to Rondon et al. (23) with slight modification as described earlier (32). In brief, cytoplasmic extracts (15 µg protein) were incubated at 30°C for 10 min with excess 1.5 ng 32P-labeled RNA probe (see above) in a reaction mixture containing 10% glycerol and (in mM) 12 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.9), 15 KCl, 0.25 EDTA, 0.25 diethiothreitol, and 5 MgCl₂, as well as E. coli RNA (200 ng/µl) in a total volume of 15 µl. The reaction mixtures were placed on ice after ribonuclease T1 treatment (0.6 units, 37°C, 20 min) and irradiated in open tubes with UV light (0.12 J) for 5 min in a Stratalinker chamber (Stratagene). After being incubated in Laemmli sample-loading buffer at 95–100°C for 3 min, the reaction mixtures were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels together with prestained molecular-weight markers (Sigma Chemical). The gels were dried and placed in contact with Kodak X-Omat film to obtain the autoradiogram.

Statistics. Comparison of the values among different groups was performed with analysis of variance. If a significant difference was found, the Tukey-Kramer multiple-range test was employed. For protein concentration, a two-tailed Student’s t-test was employed. P = 0.05 was designated as significant. Values are expressed as means ± SE.

RESULTS

Soleus muscle wet weight and total RNA concentration (µg RNA/mg wet wt) were the same as control values, and the soleus muscle-to-body weight ratio was increased by 39% (P < 0.05) at the end of the 7-day fixation of the muscle in a lengthened position (ankle is immobilized in dorsiflexion) (Table 1). However, soleus muscle wet weight, total RNA concentration, and muscle-to-body weight ratio were 55, 29, and 44% less (P < 0.05), respectively, than control after 7 days of fixation in a shortened position (plantar flexion) (Table 1).

Although Babij and Booth (2) previously reported that fixation of the soleus muscle in a shortened position for 7 days did not alter its protein concentration, similar data for a 7-day fixation of rat muscle in a lengthened position have not been previously published. An additional set of rats (n = 5) was employed to obtain this information. Fixation in a lengthened position did not alter soleus muscle protein concentration (183.1 ± 6.3 mg/g wet wt for control; 160.3 ± 9.0 mg/g wet wt for 7-day fixation; P = 0.07, two-tailed Student’s t-test) and content (15.0 ± 1.1 mg/muscle for control; 12.9 ± 0.8 mg/muscle for fixation; P = 0.17). Previously, Jokl and Konstadt (17) reported that myofibrillar and sarcoplasmic protein concentration were unchanged in cat fast- and slow-twitch skeletal muscle that had been immobilized in the lengthened position for 4 wk.

Cytochrome c mRNA was less in soleus muscles fixed in either a shortened or lengthened position. Cytochrome c mRNA per microgram RNA was 47 and 65% less (P < 0.01) in shortened and lengthened positions, respectively, after 7 days of immobilization compared with the control group of the same age (Table 1, Fig. 1). Cytochrome c mRNA concentration per milligram so-

Table 1. Cytochrome c mRNAs in soleus muscles fixed in lengthened or shortened position for 7 days

<table>
<thead>
<tr>
<th>Position</th>
<th>Body Wt, g</th>
<th>Wet Wt, mg</th>
<th>Wet Wt-to-Body Wt Ratio</th>
<th>Total RNA, mg/g</th>
<th>Cytochrome c mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IOD/µg RNA</td>
</tr>
<tr>
<td>Control</td>
<td>154 ± 2</td>
<td>628 ± 5.3</td>
<td>0.41 ± 0.01</td>
<td>1.7 ± 0.1</td>
<td>0.38 ± 0.023</td>
</tr>
<tr>
<td>Shortened (plantar flexion)</td>
<td>121 ± 1*</td>
<td>283 ± 2.1*</td>
<td>0.23 ± 0.01*</td>
<td>1.2 ± 0.1*</td>
<td>0.204 ± 0.051*</td>
</tr>
<tr>
<td>Lengthened (dorsiflexion)</td>
<td>114 ± 4*</td>
<td>646 ± 4.0*</td>
<td>0.57 ± 0.01*</td>
<td>1.9 ± 0.3*</td>
<td>0.133 ± 0.011*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Muscle weights for each animal were average of both sides. For cytochrome c mRNA analysis, 2 soleus muscles from a rat were pooled for an observation (control and lengthened groups), and 4 soleus muscles from 2 rats were pooled for a single observation (shortened group). For control and lengthened groups, n = 5; for shortened group, n = 10 rats. Average body weight and soleus muscle weights of 2 rats pooled for mRNA determination were used to calculate body and muscle weights. IOD, integrated optical density units. *P < 0.01 from control; †P < 0.05 from shortened group; ‡P < 0.01 from shortened group.
leus wet weight was 62 and 72% less (P < 0.01) in shortened and lengthened positions, respectively, compared with the control group (Table 1). Cytochrome c mRNA content per whole soleus muscle was 82 and 70% less (P < 0.01) in shortened and lengthened positions, respectively, than the control group (Table 1).

To determine whether the observed phenomena are only specific to soleus muscles, we performed the measurements on the TA muscles. The TA muscle wet weight was 37% (P < 0.05) and 13% (P < 0.01) less when it was immobilized in a shortened position (dorsiflexion) and lengthened position (plantar flexion), respectively, compared with control values (Table 2). However, fixation of the TA muscle in a lengthened position maintained the muscle-to-body weight ratio.

In a separate set of rats (n = 5), fixation in a lengthened position did not alter TA muscle protein concentration (219.7 ± 1.2 mg/g wet wt for control; 211.1 ± 4.1 mg/g wet wt for 7-day fixation; P = 0.08) but decreased protein content (76.0 ± 2.5 mg/muscle for control; 58.8 ± 1.3 mg/muscle for fixation; P < 0.05). Goldspink (12) found that the protein content of the rat extensor digitorum longus (EDL) muscle was the same as in an age-matched control after 2, 4, and 7 days of immobilization in the lengthened position. Lengthening of the rabbit EDL by immobilization for 3 days did not alter its protein concentration, which indicates that wet weight reflects protein content in this animal (13). Total RNA concentration (µg RNA/mg wet wt) was the same as the control values at the end of the 7-day fixation.

Table 2. Cytochrome c mRNAs in tibialis anterior muscles fixed in lengthened or shortened position for 7 days

<table>
<thead>
<tr>
<th>Position</th>
<th>Body Wt, g</th>
<th>Wet Wt, mg</th>
<th>Wet Wt-to-Body Wt Ratio</th>
<th>Total RNA, mg/g</th>
<th>Cytochrome c mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140 ± 2</td>
<td>587 ± 48</td>
<td>4.2 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>0.304 ± 0.043</td>
</tr>
<tr>
<td>Lengthened (plantar flexion)</td>
<td>119 ± 3*</td>
<td>509 ± 33*</td>
<td>4.8 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>0.182 ± 0.024†</td>
</tr>
<tr>
<td>Shortened (dorsiflexion)</td>
<td>118 ± 4*</td>
<td>369 ± 40†‡</td>
<td>3.1 ± 0.2†‡</td>
<td>1.3 ± 0.1</td>
<td>0.209 ± 0.019†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5). Two muscles from a rat were pooled for each observation of muscle weight, RNA, and cytochrome c mRNA. *P < 0.05 from control; †P < 0.01 from control; ‡P < 0.05 from lengthened group.
in both shortened and lengthened TA muscles (Table 2). Cytochrome c mRNA levels were less than the control values in the TA muscles immobilized in both the shortened and lengthened positions as normalized by three methods. Cytochrome c mRNA (integrated optical density unit per microgram of RNA) was 31 and 40% less (P < 0.01) in shortened and lengthened positions, respectively, after 7 days of immobilization compared with controls of the same age (Table 2). Cytochrome c mRNA per milligram wet TA weight was 36 and 32% less (P < 0.01) in shortened and lengthened positions, respectively, than in the controls (Table 2). Cytochrome c mRNA per whole TA muscle was 59 and 40% less (P < 0.01) in shortened and lengthened positions, respectively, compared with control group values (Table 2). These data again demonstrate that muscle atrophy during immobilization can be attenuated by lengthening the muscle, but cytochrome c mRNA levels decrease as long as the muscle is immobilized.

No consistent change in cytoplasmic protein interaction with a 116-base riboprobe (+1,337 to +1,452) from the 3′-UTR of rat somatic cytochrome c mRNA in cytoplasmic extracts was noted from soleus muscles fixed for 7 days in either lengthened or shortened position (Fig. 2). Similar observations were observed in TA muscle (data not shown).

**DISCUSSION**

When soleus and TA muscles were immobilized in lengthened position, cytochrome c mRNA levels per whole soleus and TA muscles were 30 and 60%, respectively, of control. In contrast, total protein per whole soleus and TA muscles was 86 and 77%, respectively, of control. Thus fixation of muscle in a lengthened position not only did not prevent loss of cytochrome c mRNA, but the decrease in cytochrome c mRNA predicts a decline in cytochrome c protein that is greater than total protein. Although the attenuation of muscle atrophy when muscles are lengthened in immobilized limbs confirms previous reports (3, 12, 28, 29), the observation that cytochrome c mRNA content is not maintained in muscles immobilized in a lengthened position is new. The disassociation of muscle mass from the loss of cytochrome c mRNA supports the hypothesis of differential gene regulation of mitochondrial and contractile protein pools by different cell signals. Examples are that resistance training enlarges muscle mass whereas mitochondrial concentration remains constant (15), endurance training increases mitochondrial concentration whereas muscle mass is unaltered (6), and muscle mass decreases whereas mitochondrial density increases during chronic stimulation (30). The hypothesis that mitochondrial and contractile proteins are modulated by different signaling pathways from contractile activity deserves further study.

Results of the present study support the idea that neither passive stretch nor isometric contractions can maintain the concentration of mitochondria in skeletal muscle in immobilized limbs. Fournier et al. (11) found that integrated electromyograph (EMG) was unchanged when the soleus muscle was fixed in a lengthened position during immobilization. This EMG activity must reflect isometric contraction because the joints were fixed. Hnık et al. (14) found similar results for the soleus muscle and extended these observations to the TA. The fixation of the ankle in neither plantar flexion nor dorsiflexion had any appreciable effect on EMG activity in the TA. Hnık et al. (14) concluded that, because immobilization in the lengthened position maintained, but did not increase, EMG activity in either the soleus or TA muscle, passive stretch, rather than EMG activity, appeared to be the factor mainly responsible for lessening atrophy of muscles fixed in the lengthened position. Previous reports indicate a direct correlation exists between duration of low-intensity isotonic contractions and cytochrome c protein concentration in skeletal muscle (8, 10). Hindlimb unloaded muscles undergo random isotonic contractions. Because it has previously been shown that cytochrome c mRNA per whole soleus muscle was decreased 54, 45, and 61% by 7 days of hindlimb unloading, limb immobilization of the soleus muscle in a shortened position, and denervation, respectively (2), the isotonic contractions occurring in the hindlimb-unloaded muscle are insufficient in duration to maintain cytochrome c mRNA.

Because muscles with more contractile activity have higher concentrations of cytochrome c mRNA and less RNA-protein interaction in the 3′-UTR of cytochrome c mRNA as determined by gel mobility shift and UV cross-linking assays (32), we hypothesized that decreasing loaded isotonic contractions of the soleus muscle

![Fig. 2. RNA-protein interaction in the 3′-untranslated region of cytochrome c mRNA. Equal amounts of protein (15 µg) from whole soleus muscle homogenates were incubated with 1.5 ng of 3P-labeled riboprobe. Autoradiogram from ultraviolet cross-linking assay with radiolabeled riboprobe for 116 bases (+1,337 to +1,452) of the 3′-untranslated region of cytochrome c mRNA. Molecular masses (in kDa; left) were estimated from prestained markers (Sigma Chemical). Groups were control, shortened (plantar flexion) for 7 days, and lengthened (dorsiflexion) for 7 days. FP, free probe.](https://jap.physiology.org/)
would increase this RNA-protein interaction. This hypothesis did not hold up. Protein interaction with the 3'-UTR was not changed by fixation of the soleus muscle in either a shortened or lengthened position. Thus the decrease in cytochrome c mRNA was not due to the increase in RNA-protein interaction in the 3'-UTR of cytochrome c mRNA. In summary, passive lengthening of the immobilized soleus muscle does not prevent the loss of cytochrome c mRNA, even though loss in its muscle mass was attenuated; and decreased cytochrome c mRNA levels in the immobilized muscles are not due to increased RNA-protein interaction in the 3'-UTR of cytochrome c mRNA. The greater losses of cytochrome c mRNA (70%) than of total protein (14%) in the whole soleus muscle during immobilization in a lengthened position underscores that countermeasures to atrophy in limb immobilization must include specific exercises to signal increases in both contractile and mitochondrial gene expression.

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REFERENCES