Cytochrome c transcriptional activation and mRNA stability during contractile activity in skeletal muscle

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Freyssenet, Damien, Michael K. Connor, Mark Takahashi, and David A. Hood. Cytochrome c transcriptional activation and mRNA stability during contractile activity in skeletal muscle. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E26–E32, 1999.—We evaluated contractile activity-induced alterations in cytochrome c transcriptional activation and mRNA stability with unilateral chronic stimulation (10 Hz, 3 h/day) of the rat tibialis anterior (TA) muscle for 1, 2, 3, 4, 5, and 7 days (n = 3–11/group). Transcriptional activation was assessed by direct plasmid DNA injection into the TA with a chloramphenicol acetyltransferase (CAT) reporter gene linked to 326 bp of the cytochrome c promoter. Cytochrome c mRNA in stimulated muscles increased by 1.3- to 1.7-fold above control between 1 and 7 days. Cytochrome c protein was increased after 5 days of stimulation to reach levels that were 1.9-fold higher than control by 7 days. Cytochrome c mRNA stability, determined with an in vitro decay assay, was greater in stimulated TA than in control between 2 and 4 days, likely mediated by the induction of a cytosolic factor. In contrast, cytochrome c transcriptional activation was elevated only after 5 days of stimulation when mRNA stability had returned to control levels. Thus the contractile activity-induced increase in cytochrome c mRNA was due to an early increase in mRNA stability, followed by an elevation in transcriptional activation, leading to an eventual increase in cytochrome c protein levels.

cell-free messenger ribonucleic acid decay; chronic stimulation; direct gene injection; mitochondrial biogenesis

IT IS WELL ESTABLISHED that mitochondrial biogenesis can be elicited in skeletal muscle in response to sustained contractile activity (10, 12). Increases in the volume of skeletal muscle mitochondria (9, 20), as well as concomitant elevations in the activity of many mitochondrial enzymes, occur in response to increased muscle activity (8, 13). These changes occurring at the protein level of expression are also accompanied by elevations in mRNAs derived from both the nuclear and the mitochondrial genomes (14, 32). Alterations in gene transcription and/or mRNA stability could potentially explain these increases. However, the relative contribution of these processes in response to contractile activity has not been established.

In the present study, we used the expression of cytochrome c as a representative model of the possible adaptations in mRNA turnover that transpire during chronic stimulation. Cytochrome c is a nuclear-encoded mitochondrial protein involved in electron transport between complexes III and IV of the mitochondrial respiratory chain. The sequence of the cytochrome c gene has been extensively studied, and both the coding region (24) and the upstream regulatory elements have been determined (11). In skeletal muscle, cytochrome c mRNA and protein levels are known to be upregulated in parallel with changes in contractile activity (8, 36). Recently, it has been shown that continuous contractile activity (24 h/day) induced an alteration in RNA-protein interactions at the 3′ end of the cytochrome c mRNA, suggesting that increases in skeletal muscle cytochrome c mRNA expression may be at least partially mediated by changes in message stability (36). To further investigate this hypothesis, we evaluated both cytochrome c transcriptional activation and mRNA degradation with a physiologically relevant contraction (3 h/day) and recovery period stimulation paradigm. To measure transcriptional activation, we used direct plasmid DNA injection into skeletal muscle. A number of studies have shown that promoter-reporter chimeric gene constructs are successfully taken up and expressed in skeletal muscle (30, 33). Transcriptional activation can be subsequently determined by measuring the expression of the protein encoded by the reporter gene. To evaluate cytochrome c mRNA degradation, we utilized a cell-free mRNA decay system (21) with extracts derived from stimulated and nonstimulated control skeletal muscle. Thus measurements of transcriptional activation as well as mRNA stability were used to interpret the time course changes in cytochrome c mRNA expression during chronic contractile activity.

METHODS

Animal care and surgery. Male Sprague-Dawley rats (283 ± 5 g) were housed individually in a temperature-controlled room (21°C) with a 12:12-h light-dark cycle and were allowed food and water ad libitum. A smaller group of animals was used for a separate mRNA stability experiment (352 ± 14 g; n = 6). Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg body wt), and platinum electrodes (Med-Wire, Leico Industries, New York, NY) were surgically implanted on both sides of the common peroneal nerve of the left hindlimb as previously described (28). This procedure was used to stimulate both the tibialis anterior (TA) and the extensor digitorum longus (EDL) muscles. The contralateral nonstimulated limb served as an internal control. Animals subject to this protocol are able to locomote freely within the cage and are free to eat and drink in unaffected fashion.

Plasmids. The plasmid constructs (pRC4CAT/-326 and -66) used to assess cytochrome c transcriptional activation were...
The pRC4CAT/-326 construct contains sequences of the rat cytochrome c promoter, which include 326 bp upstream of the transcription start site, linked to a chloramphenicol acetyltransferase (CAT) reporter gene. This construct has been previously shown to give full cytochrome c promoter activity in COS-1 cells (11). The pRC4CAT/-66 construct, which represents the minimal promoter region (11), was used in initial studies for comparison to the promoter activity in COS-1 cells (11). The pRC4CAT/-66 chloramphenicol acetyltransferase (CAT) reporter gene. This bp upstream of the transcription start site, linked to a Chicago, IL). The pRC4CAT/-326 construct contains se-

corresponding to the cytochrome c previously (28). Six hours after the cessation of stimulation, designed in part to evaluate the effect of continuous contrac-
lateral limbs, weighed, and frozen. In a separate experiment time period (21 h later), animals were anesthetized, and TA

and 7 days (27, 29). On the day after the indicated stimulation stimulated (10 Hz, 0.1-ms duration, 3 h/day) for 1, 2, 3, 4, 5,

the plasmid DNA injections, TA and EDL muscles were chronic stimulation.

in a final volume of 100 µl. The incision was then sutured, and animals were allowed to recover for 2 days before the onset of chronic stimulation.

Stimulation protocol and tissue sampling. Two days after the plasmid DNA injections, TA and EDL muscles were stimulated (10 Hz, 0.1-ms duration, 3 h/day) for 1, 2, 3, 4, 5, and 7 days (27, 29). On the day after the indicated stimulation time period (21 h later), animals were anesthetized, and TA muscles were removed from both the stimulated and contra-

lateral limbs, weighed, and frozen. In a separate experiment designed in part to evaluate the effect of continuous contrac-
tile activity on cytochrome c mRNA stability alone, animals were stimulated at 10 Hz for 10 days (24 h/day), as done previously (28). Six hours after the cessation of stimulation, bilateral TA muscles were removed, frozen, and stored. All muscles were then powdered and stored in liquid N₂ until required for analyses.

RNA analyses. Total RNA was isolated from frozen muscle powders (50–70 mg) as previously described (6). RNA concentra-
tion and purity were determined by ultraviolet photomet-
try at 260 and 280 nm, respectively. Total RNA (6 µg) was then separated on a denaturing formaldehyde-1% agarose gel and transferred overnight to a nylon membrane (Hybond N, Amersham, Mississauga, Canada). Cytochrome c and 18S rRNA radiolabeled cDNAs were generated by random primer labeling in the presence of [α-32P]dCTP (Amersham). After the removal of unincorporated nucleotides, label incorpora-
tion was determined by Cerenkov counting. Blots were prehy-
bridized overnight (42°C), and the membranes were subse-
quenty hybridized overnight at 42°C with the appropriate radiolabeled cDNA probe (2 × 10⁶ counts/min) as done previously (6). The blots were then washed for 2 × saline-sodium citrate (SSC; 1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS, and subsequently washed 3 × 10 min at room temperature with 2 × SSC, 0.1% SDS, followed by a 15-min wash at 50°C in 0.1 × SSC, 0.1% SDS. The blots were quantified by electronic autoradiography (Instantimagerr, Packard), which measures the total radioactivity in the region of the cytochrome c mRNA. In this case, all three bands corresponding to the cytochrome c mRNA species were quan-
tified. Blots were then corrected for uneven loading with subsequent probing with a cDNA encoding 18S rRNA.

Tissue extraction for protein assays and immunoblotting. Powdered tissues (20–25 mg) were diluted 40-fold (wt/vol) in 0.1 M KH₂PO₄ buffer (pH 7.2) containing 2 mM EDTA and sonicated (8 × 10 s) on ice. Samples were then centrifuged for 6 min in a Microfuge for 4°C. The supernatants were removed, and protein concentration was determined photometrically (2).

SDS gel electrophoresis and immunoblotting. Muscle protein extracts (75 µg) were applied to one-dimensional SDS polyacrylamide gels (12.5% [wt/vol]) and electrophoresed overnight. The separated proteins were then electrotransferred to nitrocellulose membranes (Hybond C, Amersham) and incubated overnight with a rabbit anti-rat cytochrome c polyclonal antibody (1:500; Ref. 25). The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1,000), allowing visualization of the antigen by a standard color reaction. The immunoblots were then quanti-
fied by laser densitometry (27). CAT and β-galactosidase activities. Muscle powders (100–

125 mg) were diluted threefold (wt/vol) in 0.25 M Tris (pH 7.9), subsequently frozen in an ethanol-dry ice bath, and thawed at 37°C three times. The resulting homogenates were then centrifuged in a Microfuge for 5 min at 4°C, and the supernatants were used as muscle extracts for CAT and β-galactosidase activities. To assess CAT activity, 10 µl of muscle extract were mixed with 4 µl of 10 mM acetyl-CoA and 8.6 µl (0.215 µCi) of [3H]chloramphenicol (55 mCi/mmol; Amersham). The mixture was adjusted to a final volume of 40 µl with 0.25 M Tris (pH 7.9) and was subsequently incubated at 37°C for 3.5 h. Chloramphenicol was then extracted from the reaction mixture with ethyl acetate, dried in a vacuum dessicator, and resuspended in 30 µl of ethyl acetate. Samples were applied to TLC plates, and the acetylated and nonacety-

lated forms of chloramphenicol were separated for 30 min at room temperature with chloroform-methanol (95:5% [vol/vol]) as the mobile phase. Signals were then visualized and quantified by electronic autoradiography (Instantimagerr, Packard). To assess β-galactosidase activity, 20 µl of the muscle extracts were combined with 130 µl of water and an equal volume (150 µl) of assay buffer, consisting of 120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercapto-

toenthanol and 1.18 mM o-nitrophenyl-β-D-galactopyranoside. The reaction mixture was then incubated at 37°C for 2.5 h, and β-galactosidase activity was determined photometrically at 420 nm. Reactions containing extracts derived from muscles that had not been transfected with the β-galactosidase con-
struct possessed an endogenous enzyme activity (7) ranging from 0.045 to 0.069 absorbance U/h. There also appeared to be a modest effect of stimulation on endogenous β-galactosidase activity, because this activity was elevated by 25–30% from day 1 to day 7 (results not shown). Thus, to use β-galactosi-
dase expression to correct CAT activity for transfection efficiency, we calculated the actual transfected β-galactosi-
dase activity by subtracting the endogenous activity from the total activity measured during the time course of the experi-

ments. In vitro mRNA decay. Protein extracts from stimulated and nonstimulated control EDL muscles were prepared according to previously published protocols (15, 36), with some modifications. Briefly, skeletal muscle powders (50–100 mg) were homogenized (Ultra Turrax, 7-mm probe) 3 × 10 s (70% maximum) in 1 ml of homogenization buffer comprised of 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM HEPES, 0.5 mM 1,4-dithiothreitol, and 0.5 mM phenylmeth-

sulfonyl fluoride. These homogenates were then centrifuged
for 15 min at 5,000 g (Beckman, Avanti J-25) at 4°C. The resulting supernatant fractions were then subjected to further centrifugation at 15,000 g (4°C) for 15 min, and the resultant postmitochondrial supernatant (S15) was transferred to a sterile Eppendorf tube. Protein concentrations of the S15 fractions from stimulated and control muscles were determined photometrically (2). Total RNA (30 or 60 µg) from stimulated or control EDL muscles was incubated with 60 or 200 µg of S15 extract obtained from the same muscles in a 300-µl reaction volume at 37°C. Aliquots were removed at various times as indicated in Fig. 1–5, and the RNA was extracted with a phenol-chloroform-isooamylic alcohol extraction procedure. The RNA was then precipitated at −70°C for 1 h and subsequently pelleted, dried, and resuspended in 10 µl of sterile H2O. The RNA was separated on a 1% agarose gel, resulting supernatant fractions were then subjected to further procedures. The RNA was separated on a 1% agarose gel, and fixed to the membrane with ultraviolet light. These membranes were probed with [32P]dCTP-labeled cDNAs specific for cytochrome c mRNA, and signals were quantified with electronic autoradiography (Instantimager, Packard).

Statistical analyses. The effects of chronic stimulation on muscle mass, total protein, total RNA, cytochrome c mRNA, and cytochrome c protein levels were evaluated with paired t-tests. Changes in cytochrome c transcriptional activation and mRNA stability after increased contractile activity were determined with two-way ANOVA, followed by Tukey’s post-hoc test. All values are presented as means ± SE, and differences were considered to be statistically significant at the 0.05 level of confidence.

RESULTS

Body mass, TA muscle weights, total protein, and total RNA concentrations. Chronic contractile activity had no effect on body mass or TA muscle mass over the 7-day time period (Table 1). Control TA muscle mass averaged 485.9 ± 12.8 mg (n = 43). Total protein concentration in control muscle averaged 84.7 ± 4.4 mg/g wet wt (n = 27) and was not influenced by contractile activity. The total RNA concentration in nonstimulated control TA muscle was 1,846.2 ± 77.2 µg/g (n = 34) and was not significantly affected by the simulation protocol (Table 1).

Cytochrome c mRNA and protein levels. Chronic contractile activity induced an increase in steady-state cytochrome c mRNA content in the TA muscle, from 1.3-fold on day 1 to 1.7-fold between days 5 and 7 (P < 0.05; Fig 1A and C). The 1.5-fold increases apparent at days 2 and 4 tended toward, but did not attain, statistical significance (0.05 < P < 0.1). There was no effect of contractile activity on 18S rRNA levels, as reported previously (27). The time course of the stimula-

### Table 1. Body mass, tibialis anterior muscle mass, total protein, and total RNA concentrations during chronic stimulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Days of Stimulation</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>255 ± 17</td>
</tr>
<tr>
<td>Muscle mass (S/C)</td>
<td>1.01 ± 0.03</td>
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<tr>
<td>Total protein (S/C)</td>
<td>0.92 ± 0.05</td>
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<tr>
<td>Total RNA (S/C)</td>
<td>1.35 ± 0.18</td>
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Values are means ± SE; n = 4–10 rats/group. Tibialis anterior muscle mass, total protein, and total RNA concentrations are expressed as ratios of levels obtained in stimulated (S) muscles to those obtained in nonstimulated control (C) muscles (S/C).
Cytochrome c transcriptional activation. Initial studies were performed with control, unstimulated TA muscles to confirm that transcriptional activation of the cytochrome c promoter would occur after injection with the cytochrome c DNA constructs. Transcriptional activation of the pRC4CAT/-66 construct was only marginally above background levels of detectability (n = 8). In contrast, CAT activity driven by the pRC4CAT/-326 construct was much higher than background levels, and it was found to progressively increase over time after injection, even as much as 5 wk later (results not shown). These initial studies confirmed the effectiveness of our injection protocol, verified the stability of the transcriptional activation product (CAT) in our injected muscles, and led us to the use of the pRC4CAT/-326 construct in evaluating the effect of contractile activity on cytochrome c transcriptional activation.

A time-dependent increase in cytochrome c promoter-driven CAT activity was evident over the 7-day period in nonstimulated TA muscle. An approximate fourfold accumulation of corrected CAT activity above that found at day 1 was evident between days 4 and 7 of the experimental protocol (Fig. 2B; n = 27). In the stimulated muscle, an increase in CAT activity was only evident after 5 days of contractile activity, reaching values that were 2.1-fold higher (P < 0.05) than those measured in contralateral, nonstimulated TA muscles (Figs. 2, A and B; n = 11).

Cytochrome c mRNA stability. In establishing the in vitro mRNA decay assay conditions, we first showed that mRNA stability was unaffected in the presence of the individual components of the decay reaction. We also documented that mRNA levels progressively decreased as a function of incubation time (5, 10, 20, and 40 min) in the presence of cytosol and that decay was linear with the amount of cytosolic fraction added (20, 40, and 66 µg protein/lane; Connor and Hood, unpublished observations). The assay conditions chosen (10 and 20 min; 20 µg protein/lane) were based on the observation that mRNA stability was no longer evident after 5 days of stimulation, when rates of degradation in the stimulated muscle were equivalent to the cytosol-induced degradation of cytochrome c mRNA from control muscle (Fig. 4, A, n = 27). In the stimulated muscle (lanes 1-3) was degraded at a slower rate than cytochrome c mRNA from control muscle (lanes 4-6 and B). This contractile activity-mediated increase in cytochrome c mRNA stability was no longer evident after 5 days of stimulation, when rates of degradation in the stimulated muscle were equivalent to the cytosol-induced degradation of cytochrome c mRNA from control muscle (Fig. 4, A, n = 27).
(lanes 1-3 vs. lanes 4-6) and B). These results at 5 days were also similar to rates of cytochrome c mRNA decay at both 1 and 7 days (not shown).

In a separate subset of animals, we further evaluated the effect of a cytosolic fraction in mediating cytochrome c mRNA stability under conditions of continuous contractile activity (24 h/day for 10 days), similar to that used recently (36). This treatment resulted in a 1.9-fold increase in cytochrome c mRNA in the stimulated muscle. In these decay reactions, cytosolic fractions obtained from both stimulated and control muscles were incubated with RNA isolated from control muscle. Cytochrome c mRNA was more stable in the presence of cytosolic fraction derived from the muscle subject to continuous chronic contractile activity (Fig. 5). The $t_1/2$ value (26 min) was 88% higher in the presence of the control cytosol (14 min). Taken together, these data are consistent with the idea that a cytosolic component is induced in response to chronic contractile activity and that it acts to reduce the degradation rate of cytochrome c mRNA. In addition, this effect appears to be at least partially mRNA specific, because no effect of contractile activity (for 2–4 days, 3 h/day) on $\alpha$-actin mRNA stability was observed (Connor and Hood, unpublished observations).

**DISCUSSION**

Contractile activity is a potent stimulus for the induction of mitochondrial biogenesis in skeletal muscle (for reviews, see Refs. 10 and 12) and thus serves as a good experimental model to study the underlying mechanisms involved in organelle synthesis and turnover. In the present study, contractile activity was induced with a chronic low-frequency electrical stimulation protocol (10 Hz, 3 h/day), which has been shown to lead to mitochondrial phenotypic alterations, as well as increases in muscle mitochondrial content (27, 29). To document some of the underlying mechanisms involved in these adaptations, we chose to measure cytochrome c protein and mRNA levels, as well as the attendant processes of gene transcription and mRNA stability. The results indicate that increases in cytochrome c expression brought about by chronic contractile activity are due to time-dependent adaptations, which appear to involve an increase in mRNA stability, followed by a subsequent increase in transcriptional activation.

The application of a cell-free mRNA decay assay (21) to measure mRNA degradation in the presence of a cytosolic fraction permitted the conclusion that the early increases in cytochrome c mRNA expression were due to a change in mRNA stability. The mRNA decay assay employed appears to be sensitive enough to measure subtle alterations in mRNA stability, even when studying mRNAs possessing relatively low rates of turnover, as found in skeletal muscle (6). The cellular events mediating the stabilization of cytochrome c mRNA as a result of contractile activity likely involve
cytochrome c expression in chronically stimulated muscle

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