Effect of chronic contractile activity on mRNA stability in skeletal muscle

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Lai RY, Ljubicic V, D’souza D, Hood DA. Effect of chronic contractile activity on mRNA stability in skeletal muscle. Am J Physiol Cell Physiol 299: C155–C163, 2010. First published April 7, 2010; doi:10.1152/ajpcell.00523.2009.—Repeated bouts of exercise promote the biogenesis of mitochondria by multiple steps in the gene expression patterning. The role of mRNA stability in controlling the expression of mitochondrial proteins is relatively unexplored. To induce mitochondrial biogenesis, we chronically stimulated (10 Hz; 3 or 6 h/day) rat muscle for 7 days. Chronic contractile activity (CCA) increased the protein expression of PGC-1α, c-myc, and mitochondrial transcription factor A (Tfam) by 1.6-, 1.7-, and 2.0-fold, respectively. To determine mRNA stability, we incubated total RNA with cytosolic extracts using an in vitro cell-free system. We found that the intrinsic mRNA half-lives (t1/2) were variable within control muscle. Peroxisome proliferator-activated receptor-γ, coactivator-1α (PGC-1α) and Tfam mRNAs decayed more rapidly (t1/2 = 22.7 and 31.4 min) than c-myc mRNA (t1/2 = 99.7 min). Furthermore, CCA resulted in a differential response in degradation kinetics. After CCA, PGC-1α and Tfam mRNA half-lives decreased by 48% and 44%, respectively, whereas c-myc mRNA half-life was unchanged. CCA induced an elevation of both the cytosolic RNA-stabilizing human antigen R (HuR) and destabilizing AUF1 (total) by 2.4- and 1.8-fold, respectively. Increases in the p37AUF1, p40AUF1, and p45AUF1 isoforms were most evident. Thus these data indicate that CCA results in accelerated turnover rates of mRNAs encoding important mitochondrial biogenesis regulators in skeletal muscle. This adaptation is likely beneficial in permitting more rapid phenotypic plasticity in response to subsequent contractile activity.

mitochondrial biogenesis; PGC-1α; exercise; mitochondrial transcription factor A; AU-rich element

SKELETAL MUSCLE exhibits dynamic plasticity in response to functional demands. With chronic contractile activity (CCA), muscles undergo a wide variety of biochemical and physiological adaptations (21, 39). For example, regular endurance exercise undertaken over a number of weeks leads to the biogenesis of mitochondria, eliciting a sequential phenotypic fiber type transition from glycolytic to oxidative metabolism (4, 21, 22). This process is orchestrated, in part, by the well-characterized key regulatory factor, peroxisome proliferator-activated receptor-γ, coactivator-1α (PGC-1α) (3a, 21, 61). Specifically, PGC-1α coactivates transcription factors to induce the expression of mitochondrial transcription factor A (Tfam), a nuclear gene product that is involved in the transcription and replication of mitochondrial DNA (mtDNA) (51, 52).

In response to exercise, mRNA and protein levels of PGC-1α and Tfam are elevated (2, 17, 43–45, 54). Increases in steady-state mRNA levels as a result of a stimulus such as exercise are the product of both synthesis (transcription) and degradation (mRNA stability). Thus the relative contribution of both of these processes plays an important role in phenotypic adaptations (16). However, the impact of changes in mRNA stability for muscle mitochondrial adaptations has been relatively unexplored.

When compared with other biological molecules, the distinguishing property of mRNA is its high rate of turnover, which is tissue specific (8). Of particular interest is the class of mRNA bearing AU-rich elements (AREs) in the 3′-untranslated region (−UTR) (6, 7, 11, 12). The physical interaction of AREs with specialized RNA-binding proteins (RBPs) is a strong determinant of mRNA stability and/or translation, and hence the subsequent level of protein expression. In muscle, a pair of RBPs with reciprocal functions can act independently and synchronously to regulate the expression of labile genes (42, 46, 55, 60). Human antigen R (HuR) and the four isoforms of ARE-RNA binding factor 1 (AUF1) (p37AUF1, p40AUF1, p42AUF1, and p45AUF1) are primarily localized to the nucleus (10, 31, 64). Their export to the cytoplasm has been associated with the stabilization or destabilization of mRNA transcripts, respectively (10, 31). During muscle differentiation, HuR accumulates in the cytoplasm and interacts with mRNAs encoding MyoD, myogenin (62, 63), and acetylcholinesterase (13, 14). In contrast, the AUF1 proteins have been shown to bind to and destabilize the mRNAs encoding human angiotensin 1 receptor (47), sarcoendoplasmic reticulum calcium ATPase2a (5), and the calcitonin receptor (69). Furthermore, HuR and AUF1 have common targets, and growing evidence has demonstrated that homomultimerization of HuR and AUF1 can bind simultaneously and competitively to a single ARE (32, 47, 69). Interestingly, both HuR and the AUF1 proteins target the ARE in the c-myc 3′-UTR (18, 31). C-Myc is a transcription factor that has also been implicated in the regulation of mitochondrial biogenesis via the control of Tfam expression (29, 34).

The effect of contractile activity on the stability of mRNAs that regulate mitochondrial biogenesis has never been investigated. Thus the purposes of our study were 1) to characterize the mRNA stability of PGC-1α, c-myc, and Tfam in relation to steady-state mRNA and protein levels; 2) to investigate the presence of relevant AREs within the 3′-UTRs of PGC-1α, c-myc, and Tfam regulatory genes; and 3) to determine the effect of CCA on the expression of mRNA destabilizing/destabilizing proteins along with coincident changes in mRNA half-lives.

MATERIALS AND METHODS

Animals and in vivo chronic contractile activity protocol. All animals were housed in a temperature-controlled room (22.5 ± 0.5°C) with 12 h light (19:00–7:00) and 12 h dark (7:00–19:00) cycles and were allowed food and water ad libitum. Male Sprague-Dawley rats...
(300–400 g; Charles River, St. Constant, QC, Canada) were anesthetized with a ketamine-xylazine combination dose via intraperitoneal injection (0.2 ml/100 g body wt). The tibialis anterior (TA) muscles were removed from a subset of animals for total RNA isolation. In another set of animals (300–325 g) used for chronic contractile activity (CCA), two stimulating electrodes (Medwire, Leico Industries, New York, NY) were passed subcutaneously from the thigh and exteriorized at the back of the neck under aseptic conditions. A portable stimulator unit was placed in a plastic housing connected to the exteriorized electrode wires at the back of the neck and fastened to the back of the animal with cloth tape, as done previously (1, 36). Care was taken to ensure that the procedure did not restrict animal movement, cause discomfort, or restrict breathing. The electrodes were sutured to the underlying muscle ~1–2 mm on either side of the common peroneal nerve. The overlying hamstring muscle was sutured, the skin was stapled, and sterile ampicillin (Penbritin, Ayerst, Montreal, Canada) was injected to minimize risks of infection. In all animals, the contralateral limb was used as a nonstimulated internal control. Stimulation was adjusted at the time of electrode implantation to result in palpable contractions of the TA and extensor digitorum longus (EDL) muscles. When animals had regained ≥100% of their initial body weight and had recovered for a minimum of 1 wk, chronic stimulation (10 Hz, 0.1-ms duration) was begun for 3 h or 6 h/day for 7 days. Animals were observed to move freely about their cages during the experimental periods. After the final recovery period (21 or 18 h) on the eighth day, animals were then anesthetized with ketamine-xylazine as described above, and the EDL muscles were extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart. All extracted and frozen in liquid nitrogen. All animals were then immediately euthanized via thoracotomy and removal of the heart.

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RESULTS

Effect of CCA on whole muscle mitochondrial biogenesis. To evaluate the effect of CCA on mitochondrial content, we measured COX activity in stimulated and contralateral nonstimulated EDL muscle. COX activity in nonstimulated EDL muscle was $6.06 \pm 0.56$ U/g muscle. Seven days of stimulation resulted in an equal elevation in animals subjected to both 3 and 6 h/day conditions (unpublished data). Therefore, the results were pooled to show a 1.5-fold elevation of COX activity (Fig. 1A; $P < 0.05$). Thus our chronic stimulation protocol was effective in inducing mitochondrial biogenesis in whole muscle.

Effect of CCA on PGC-1α protein content, steady-state mRNA level, and mRNA stability. CCA resulted in an increase of PGC-1α protein content by 1.6-fold when compared with nonstimulated control muscle (Fig. 1B; $P < 0.05$). In contrast, the levels of PGC-1α mRNA were not affected by the chronic stimulation paradigm (Fig. 1C). To evaluate PGC-1α mRNA stability, we employed a cell-free mRNA decay assay, as done previously (16). PGC-1α mRNA was degraded by cytosolic ribonuclease, as incubation of total RNA in the absence of cytosolic proteins (Fig. 2A) and in the presence of a ribonuclease inhibitor (Fig. 2B) attenuated the mRNA decay. The half-life ($t_{1/2}$) of PGC-1α mRNA was 24.5 min (Fig. 2C) in the presence of cytosolic proteins isolated from control muscle. In contrast, PGC-1α mRNA stability decreased by 48% in the cytosol isolated from chronically stimulated muscle (Fig. 2C; $P < 0.05$). S12 mRNA, used as an internal control, was more stable ($t_{1/2} = 58.6$ min) than PGC-1α mRNA in the cytosol from control, nonstimulated muscle. No effect of chronic stimulation on S12 mRNA was observed (unpublished data). Therefore, these data for S12 mRNA were combined (Fig. 2C).

Effect of CCA on c-myc protein content, steady-state mRNA level, and mRNA stability. CCA resulted in an increase of c-myc protein content by 1.7-fold (Fig. 3A; $P < 0.05$). In contrast, both c-myc steady-state mRNA level and mRNA stability were unchanged in response to chronic stimulation (Fig. 3, B and C). The basal half-lives of c-myc (82.9 min) and S12 (52.6 min) mRNAs were not significantly different from each other.

Effect of CCA on Tfam protein content, steady-state mRNA level, and mRNA stability. CCA led to an increase of Tfam protein content by 2.0-fold (Fig. 4A; $P < 0.05$) but not steady-state mRNA levels (Fig. 4B). In the presence of cytosolic proteins isolated from control muscle, Tfam mRNA

Statistical analyses. Data were analyzed with GraphPad 4.0 software, and values are reported as means ± SE. Where indicated, Student’s paired $t$-test, one- or two-way analyses of variance followed by Bonferroni post hoc tests were used to determine individual differences between conditions. Results were considered to be statistically significant if $P < 0.05$ was achieved. The nonlinear regression equation $50 = 100e^{(-k_{i}t)}$ was used to determine mRNA half-lives.

Computational analysis. To identify putative HuR and AUF1 binding motifs in PGC-1α 3’-UTRs of mammals, we deployed sequence comparison of six mammalian species against human using the software ClustalW2 (33). A pairwise score was calculated as the number of identities in the best alignment divided by the number of residues compared. Gap positions were excluded. Both of these scores were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site (33).
The half-life was 31.2 min, and this was reduced by 44% to 13.6 min in chronically stimulated muscle compared with the control condition (Fig. 4C; \( P < 0.05 \)).

**Effect of CCA on RNA-binding proteins HuR and AUF1.** Analysis of each AUF1 isoform revealed that the most abundant isoform was p45\(^{\text{AUF1}}\), whereas p42\(^{\text{AUF1}}\) expression was low in fast-twitch EDL muscle. CCA resulted in the increase of stabilizing HuR (Fig. 5A; \( P < 0.05 \)) as well as destabilizing total AUF1 proteins (Fig. 5B; \( P < 0.05 \)) by 2.4- and 1.9-fold, respectively. p37\(^{\text{AUF1}}\), p40\(^{\text{AUF1}}\), and p45\(^{\text{AUF1}}\) isoforms increased in response to CCA by 2.3-, 1.7-, and 1.5-fold, respectively (Fig. 5C; \( P < 0.05 \)), when compared with the contralateral control muscle.

**Effect of CCA on mRNA stability.** A summary of the half-lives of the transcripts measured in our study is shown in Fig. 6. These half-lives are not intended to represent the decay rates as found in vivo, since they are a product of the conditions

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**C158 EFFECT OF CONTRACTILE ACTIVITY ON mRNA STABILITY IN MUSCLE**

**Fig. 2.** Effect of CCA on PGC-1\(\alpha\) mRNA stability in skeletal muscle. A: total RNA (12 \( \mu \)g) from the tibialis anterior (TA) muscle was incubated with RNAse-free isolation buffer alone for 0, 10, 20, and 40 min. PGC-1\(\alpha\) and S12 mRNA transcripts were examined by RT-PCR and EtBr-stained agarose gel electrophoresis. B: total RNA (30 \( \mu \)g) was incubated with 20 \( \mu \)g of cytosolic proteins for 0, 5, 10, 20, and 30 min in the presence or absence of a ribonuclease inhibitor. Reactions in the absence of RNA or cDNA are shown as negative controls. C: total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was reisolated and PGC-1\(\alpha\) (\( n = 5 \); \( * P < 0.05 \), CTRL vs. STIM) along with an internal control S12 (\( n = 10 \)) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins, as well as the absence of RNA or cDNA, are shown as positive and negative controls, respectively.

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**Fig. 3.** Effect of CCA on c-myc steady-state protein, mRNA levels, and mRNA stability. Animals were subjected to CCA for 7 days. A: c-myc protein, along with a loading control (acilcumin), were measured in extracts from CTRL or STIM EDL muscles (\( n = 8 \); \( * P < 0.05 \), CTRL vs. STIM). B: c-myc and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from CTRL or STIM EDL muscles. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins, as well as the absence of RNA or cDNA, are shown as positive and negative controls, respectively.

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employed in the in vitro decay assay (i.e., the ratio of cytosolic protein to total RNA). However, since the assay conditions used were identical for all transcripts evaluated, as well as in control and stimulated muscle, this information is valuable to show 1) the variability in the basal half-lives of mRNAs and 2) the effect of chronic contractile activity. The intrinsic mRNA stabilities of PGC-1α/H9251 and Tfam were less than that of c-myc and S12 (Fig. 6, P < 0.05). In addition, CCA induced a differential response on mRNA stability, which was reduced in PGC-1α/H9251 and Tfam, but no significant effect of stimulation was observed for S12 or c-myc (Fig. 6).

DISCUSSION

Repeated contractions of skeletal muscle over several weeks promote the biogenesis of mitochondria. The resulting increase in mitochondrial content in skeletal muscle leads to a preferential shift in energy utilization toward a greater dependence on oxidation phosphorylation for ATP provision. A consequence of this adaptation is that the muscle become more fatigue resistant, a characteristic favorable for health in both young and old individuals (37, 39). Therefore, understanding the molecular basis of mitochondrial biogenesis is important for our comprehension of how exercise can improve the quality of life.

Mitochondrial biogenesis requires the coordinated regulation of two distinct genomes localized in separate cellular compartments. Three important biogenesis regulators investigated in our study were PGC-1α, c-myc, and Tfam. PGC-1α coactivates genes encoding a number of transcription factors to induce the mRNA expression of NUGEMPs (21, 22, 28). One of the most important NUGEMPs is Tfam, which regulates the expression and copy number of mtDNA. The transcription factor c-myc is also involved in the regulation of Tfam (29,

![Fig. 4. Effect of CCA on mitochondrial transcription factor A (Tfam) steady-state protein and mRNA levels and mRNA stability. Animals were subjected to CCA for 7 days. A: Tfam protein, along with a loading control (GAPDH), were measured in extracts from CTRL or STIM EDL muscles (n = 8; *P < 0.05, CTRL vs. STIM). B: Tfam and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from CTRL or STIM EDL muscles. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n = 8). C: total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was reisolated and Tfam (n = 7, *P < 0.05 CTRL vs. STIM) along with an internal control S12 (n = 10) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins as well as the absence of RNA or cDNA are shown as positive and negative controls, respectively.](http://ajpcell.physiology.org/)

![Fig. 5. Effect of CCA on RNA-binding proteins, human antigen R (HuR), and ARE-RNA binding factor 1 (AUF1). Animals were subjected to CCA for 7 days. A: HuR, along with a loading control aciculin, were measured in extracts from CTRL or STIM EDL muscles (n = 8; *P < 0.05, CTRL vs. STIM). B: analyses and graphical representations of protein expression of p37^{AUF1}, p40^{AUF1}, p42^{AUF1}, p45^{AUF1}, and aciculin, the loading control are shown (n = 6, *P < 0.05, CTRL vs. STIM). C: distribution of various isoforms are shown. (n = 6; *P < 0.05 CTRL vs. STIM).](http://ajpcell.physiology.org/)
mRNA expression of both PGC-1α/H9251, c-myc, and Tfam in skeletal muscle and their intrinsic mRNA stabilities of PGC-1α and Tfam are time dependent, postexercise. They increase transiently after each period of contractile activity, contribute to the accumulation of protein, and then decline thereafter.

The present study is the first to demonstrate a differential response in mRNA degradation kinetics as a result of CCA. In the presence of cytosolic proteins isolated from stimulated muscle, the half-lives of PGC-1α and Tfam mRNAs were significantly reduced compared with the control, nonstimulated condition. In contrast, neither c-myc nor S12 mRNA stability was significantly altered following CCA. In a previous study employing the identical chronic stimulation protocol as the present study, Freyssenet et al. (13) showed that CCA resulted in upregulated cytochrome c mRNA abundance, which was attributed, in part, to an increased mRNA stability in the early stages (days 2 and 3) of the CCA paradigm. Later, by day 7, mRNA stability had returned to control levels (16). Conversely, Irrcher et al. (26) showed that contractile activity resulted in reduced Egr-1 mRNA stability in cultured myotubes. These divergent data are clearly indicative of transcript-specific adaptations to CCA. This depends on the presence or absence of mRNA-specific sequences, which support the interaction with stabilizing/destabilizing RBPs. We speculate that the short half-lives of PGC-1α and Tfam mRNAs are caused by one or more potent instability sequences, within the 3′-UTR of each transcript. The 3′-UTR of Tfam is known to contain AREs, which may serve as destabilizing cis-elements. Similarly, AREs may exist in the PGC-1α 3′-UTR, the full-length of which has yet to be completely identified empirically. Multiple AREs covering a stretch of ~600 nucleotides are found within ~3 kb downstream of the PGC-1α coding sequence, followed by a consensus poly(A) signal (Fig. 7A).
With the use of ClustalW2 software (33), sequence alignment of the human PGC-1α sequences against six mammalian species, revealed scores ≥89 (Fig. 7, B and C), suggesting that they are evolutionarily conserved and thus may dictate the mRNA turnover rate of this regulatory protein.

Since mRNA stability is determined by the interaction between intrinsic cis-elements and extrinsic trans-regulatory factors, we investigated the expression of ARE-regulatory RBPs, stabilizing HuR, and destabilizing AUF1. AUF1 consists of four isoforms with variable concentrations. The most abundant stabilizing HuR, and destabilizing AUF1. AUF1 consists of tors, we investigated the expression of ARE-regulatory RBPs, between intrinsic mRNA turnover rate of this regulatory protein.

In summary, our data show that the half-lives of mRNAs encoding transcription factors are variable in skeletal muscle. With respect to PGC-1α, we have revealed that the 3′-UTR may harbor functional regulatory sequences. Furthermore, the responsiveness of each mRNA species to extracellular stimuli was variable. CCA selectively increased the mRNA degradation rate of both PGC-1α and Tfam, two important regulatory proteins involved in mitochondrial biogenesis. We speculate that this characteristic is beneficial because, when combined with a parallel increase in transcription, this permits a higher turnover rate, and a more rapid adaptive response in the face of an imposed stress, such as acute contractile activity. In support of this, Pilgaard et al. (48) illustrated that PGC-1α mRNA levels. Under such conditions, transcription rates of PGC-1α are likely reduced, and mRNA stability may be enhanced in compensatory fashion. These ideas require experimental verification using muscle disuse models in the future.
with the potential of generating interventions to enhance exercise-induced benefits in healthy, aged, or diseased muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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