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Contractile activity-induced mitochondrial biogenesis and mTORC1

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Carter HN, Hood DA. Contractile activity-induced mitochondrial biogenesis and mTORC1. *Am J Physiol Cell Physiol* 303: C540–C547, 2012. First published June 13, 2012; doi:10.1152/ajpcell.00156.2012.— In response to exercise training, or chronic contractile activity, mitochondrial content is known to be enriched within skeletal muscle. However, the molecular mechanisms that mediate this adaptation are incompletely defined. Recently, the protein complex, mammalian target of rapamycin complex 1 (mTORC1), has been identified to facilitate the expression of nuclear genes encoding mitochondrial proteins (NUGEMPs) in resting muscle cells via the interaction of the mTORC1 components, mTOR and raptor, the transcription factor Yin Yang 1, and peroxisome proliferator-activated receptor- γ coactivator-1 α . It is currently unknown if this mechanism is operative during the increase in mitochondrial content that occurs within skeletal muscle with chronic contractile activity (CCA). Thus we employed a cell culture model of murine skeletal muscle and subjected the myotubes to CCA for 3 h per day for 4 consecutive days in the presence or absence of the mTORC1 inhibitor rapamycin. CCA produced increases in the mitochondrial markers cytochrome oxidase (COX) IV (2.5-fold), Tfam (1.5-fold), and COX activity (1.6-fold). Rapamycin-mediated inhibition of mTORC1 did not suppress these CCA-induced increases in mitochondrial proteins and organelle content. mTORC1 inhibition alone produced a selective upregulation of mitochondrial proteins (COX IV, Tfam), but diminished organelle state 3 respiration. CCA restored this impairment to normal. Our results suggest that mTORC1 activity is not integral for the increase in mitochondrial content elicited by CCA, but is required to maintain mitochondrial function and homeostasis in resting muscle.

skeletal muscle; peroxisome proliferator-activated receptor- γ coactivator-1 α ; Yin Yang 1; raptor; nuclear genes encoding mitochondrial proteins

SKELETAL MUSCLE IS AN adaptive tissue that can respond in a multitude of ways to regular exercise, or chronic contractile activity (CCA). A notable response to endurance exercise is the enrichment of muscle mitochondrial content, termed mitochondrial biogenesis (18). This adaptation leads to greater fatty acid oxidation and a reduction in mitochondrial-derived reactive oxygen species (1, 19). A coordinated series of cellular and molecular signaling events is necessary to convert the mechanical stimulus of exercise into the production of new mitochondria (25). Typically, kinases are activated in the muscle milieu and, in turn, they promote the activation of transcriptional coactivators, like peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α , through post-translational modifications (41). PGC-1 α is able to enter the nucleus and bind to transcription factors that occupy the promoters of nuclear genes encoding mitochondrial proteins (NUGEMPs) to upregulate their expression (41). However, the complete picture of the cellular and molecular mechanisms that mediate the exercise-induced increase in mitochondrial content is incompletely defined.

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In recent years, the multiprotein complex called the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) has been identified to influence mitochondrial content and function in quiescent muscle cells. mTORC1 is composed of mTOR, regulatory associated protein of TOR (raptor), mammalian lethal with SEC13 protein 8/G protein β -subunit-like protein (mLST8/G β L), proline-rich Akt substrate of 40 kDa (PRAS40) and DEP-domain-containing and mTOR-interactive protein (DEPTOR) (37, 48). Canonically, mTORC1 is regarded as a cellular rheostat for nutrient, oxygen, and stress signals and is intimately tied to the regulation of protein synthesis and autophagy (37, 48). mTORC1 also appears to be involved in regulating steady-state mitochondrial content, in that the muscle-specific deletion of mTOR, or raptor, results in reduced markers of mitochondrial content, as well as impaired mitochondrial respiration (4, 32). A transcriptional mechanism has been described for the expression of NUGEMPs with mTORC1 facilitating the interaction of the transcription factor Yin Yang 1 (YY1) with the transcriptional coactivator PGC-1 α (9). The expression of NUGEMPs in resting C2C12 myotubes was reduced with the acute addition of the selective mTORC1 inhibitor rapamycin (9). Rapamycin provoked the dissociation of PGC-1 α from the YY1-mTORC1 complex and impaired transcriptional activation (9). In addition, a study in human T cells demonstrated that rapamycin was able to reduce mitochondrial oxygen consumption after only 12 h of exposure (35).

However, contrasting observations have also been made regarding mTORC1 and mitochondrial regulation in lower organisms, and also in mammalian tissues. In *S. Cerevisiae*, TOR inactivation resulted in enhanced mitochondrial respiration (6, 27, 28). In addition, the ablation of raptor in murine adipose tissue produced an increase in mitochondrial respiration through the enhanced expression of uncoupling protein 1 (UCP1) (30). Finally, the treatment of rat L6 myotubes for 24 h with rapamycin provoked an increase in the reliance on fatty acid oxidation and a decrease in glucose utilization, suggesting augmented mitochondrial metabolism (38).

Given the perspectives provided above, we sought to evaluate the role of mTORC1 in maintaining mitochondrial content in skeletal muscle cells, with a particular focus on the function of mTORC1 in mediating contractile activity-induced mitochondrial biogenesis. Through the use of rapamycin to inhibit mTORC1 in murine myotubes, we demonstrate that CCA-induced mitochondrial biogenesis was not attenuated with mTORC1 inhibition. However, inhibition of mTORC1 in resting myotubes impaired mitochondrial respiration, a defect that was attenuated with the addition of CCA.

MATERIALS AND METHODS

Cell culture and chronic and acute contractile activity. C2C12 myoblasts (ATCC, Manassas, VA) were plated in six-well dishes (Sarstedt, Montreal, QC, Canada) coated with 0.1% gelatin (Sigma, St. Louis, MO) in growth media (DMEM; Sigma, St. Louis, MO) with

10% FBS (Fisher Scientific, Ottawa, ON, Canada) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and allowed to proliferate for 24 h. The cells were then induced to differentiate into myotubes through the replacement of growth media with differentiation media (DMEM with 5% heat-inactivated horse serum; Invitrogen; 1% penicillin-streptomycin). The cells were allowed to differentiate for 5 days, with media replenishment on a daily basis, and the myotubes were then subjected to electrical stimulation to induce CCA. Before the first bout of contractile activity, DMSO (Sigma) or 1 μ M rapamycin (BioShop, Toronto, ON, Canada) was added to the media. The protocol for CCA has been described previously in detail (8, 42). Briefly, customized six-well plate lids were outfitted with two platinum wires per well and were submerged into the media of plates containing fully differentiated myotubes. The plate lid was attached to a stimulator unit, and myotubes were induced to contract (9 V, 5 Hz) chronically for 3 h per day, for 4 consecutive days, with 21 h of recovery between bouts. Media containing the appropriate treatment was changed before and after each contractile bout. Samples were collected after the final 21-h recovery period. For the acute contractile activity protocol, myotubes were stimulated for 2 h (10 V, 5 Hz) and cells were either collected immediately or after a 2-h recovery period.

Myotube width quantification. Myotube images were taken through an inverted light microscope (Telaval 31; Zeiss, Germany) at 10 \times magnification using a Canon Powershot G5 Camera. Photographs were taken for all treatment conditions, and each was coded in an investigator-blind manner. The largest width was determined for each myotube in the image, and the widths were averaged from each photo. ImageJ software was used for the myotube analysis.

Mitochondrial isolation and respiration. Mitochondria were isolated from myotubes using an adapted protocol (15). Briefly, the myotubes were washed in ice-cold PBS and harvested from the plates using a cell scraper. The collected cells were subjected to homogenization with a Teflon pestle on glass, followed by differential centrifugation to yield the mitochondrial fraction. The mitochondria were resuspended in 60 μ l isolation buffer containing 10% 0.1 M Tris-MOPS, 1% EGTA-Tris, and 20% 1 M sucrose (pH 7.4). Mitochondria (50 μ l) were added to 250 μ l of VO₂ buffer containing (in mM) 250 sucrose, 50 KCl, 25 Tris base, and 10 K₂HPO₄ (pH 7.4), and oxygen consumption was measured at 30°C using a Clark electrode in the presence of 10 mM glutamate (state 4) or glutamate plus 0.44 mM ADP (state 3). Respiration rates were corrected for total protein concentration, which was measured using the Bradford assay.

Cytochrome oxidase enzymatic activity. Cells were scraped and collected on ice, sonicated 3 \times 3 s in muscle extraction buffer containing (in mM) 100 Na/KPO₄ and 2 EDTA (pH 7.2), subjected to two freeze-thaw cycles, and spun at 13,000 g for 3 min to yield the supernate (42). These extracts (50 μ l) were incubated with fully reduced horse heart cytochrome c (Sigma) for 1 min at 30°C, and the rate of oxidation was measured as the change in the rate of absorbance at 550 nm using a microplate reader (Synergy HT). Oxidation rates were corrected for protein concentration determined through the Bradford assay.

Immunoblotting. The myotubes were scraped off the plates and were collected in 1 \times passive lysis buffer (Promega, Madison, WI) fortified with protease and phosphatase inhibitors. Equal amounts of protein (50 μ g) were separated on SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare, Waukesha, WI). Membranes were incubated overnight at 4°C with the following primary antibodies: phospho-S6K1 (Thr³⁸⁹; Cell Signaling), cytochrome oxidase (COX) IV (Invitrogen), Tfam (gift from Dr. Hiroshi Inagaki), PGC-1 α (Millipore), raptor (Bethyl Laboratories), YY1 (Santa Cruz), phospho-mTOR (Ser²⁴⁴⁸; Cell Signaling), phospho-raptor (Ser⁷⁹²; Cell Signaling), phospho-AMPK (Thr¹⁷²; Cell Signaling), total-AMPK- α (Cell Signaling), phospho-Akt (Ser⁴⁷³; Cell Signaling), total-Akt (Cell Signaling), and α -Tubulin (Calbiochem). The following day, membranes were washed with 1 \times Tris-buffered saline/Tween 20 [TBST; 25 mM Tris-HCl (pH 7.5), 1 mM NaCl, and 0.01%

Tween 20] and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature. After 1 h, membranes were washed for a final time and visualized on film using enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA), as per the manufacturer's instructions. α -Tubulin was used as a loading control for most proteins. The exceptions were the nuclear fractions, where sample loading was controlled for using Histone 2B (Cell Signaling).

Cellular fractionation. Myotubes were harvested in ice-cold PBS (Sigma) and subjected to cellular fractionation using the NE-PER Nuclear and Cytoplasmic Extraction Kit as described by the manufacturer (Thermo Fisher Scientific, Rockford, IL).

Statistics. Data were expressed as means \pm SE. A two-way repeated-measures ANOVA was used to compare the groups during the chronic stimulation protocol, with Bonferroni post hoc tests for significance where appropriate. A paired *t*-test was used to compare the data for phospho-Akt. A one-way ANOVA was used for comparison of the acute stimulation data with Bonferroni post hoc tests where applicable. Significance was considered if $P < 0.05$, and Graphpad Prism 4 software was used for the analyses.

RESULTS

Indicators of mTORC1 inhibition. To determine the effectiveness mTORC1 inhibition through rapamycin treatment, we evaluated the direct downstream target S6K1. Phosphorylation of S6K1 on Thr³⁸⁹ was completely ablated in the control condition, and also after 4 days of CCA in the presence of rapamycin (Fig. 1A). Because previous literature indicated that mTORC1 inhibition also resulted in cellular atrophy, we assessed the width of the myotubes under all conditions. mTORC1 inhibition produced a significant 30% reduction in

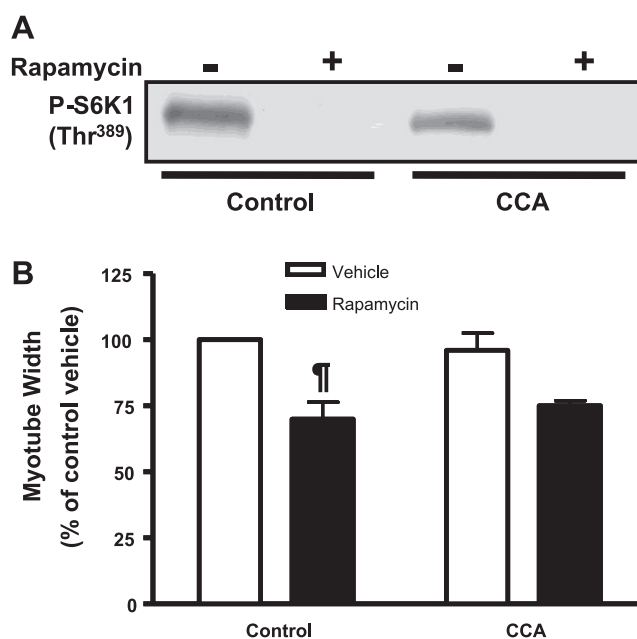


Fig. 1. Effectiveness of rapamycin treatment and myotube width. Mature myotubes were treated with 1 μ M of rapamycin or DMSO (vehicle) and exposed to a chronic contractile activity (CCA) protocol 3 h per day for 4 days. Samples were imaged and then harvested after the last recovery period. **A:** rapamycin was successful at impairing mammalian target of rapamycin complex 1 (mTORC1) signaling as determined by the lack of phosphorylation of the downstream target P-S6K1 Thr³⁸⁹. **B:** myotube width expressed as a percentage of vehicle control in the presence of vehicle or rapamycin ($\dagger P < 0.05$, rapamycin vs. vehicle control; $n = 6$ experiments).

myotube diameter; however, in the presence of CCA, the decrease in diameter was no longer significant (Fig. 1B), suggesting a modest attenuation of the atrophy in the presence of CCA. Thus we conclude from these data that our rapamycin treatment was an effective inhibitor of mTORC1 signaling.

Markers of mitochondrial content and function. We sought to determine the effects of mTORC1 inhibition on mitochondrial content and function in response to 4 days of CCA. CCA alone significantly enhanced mitochondrial content, as evidenced by a 60% increase in COX activity, and also a 2.5-fold increase in COX IV protein expression (Fig. 2, A and B). The primary transcription factor for mtDNA, Tfam, was also up-regulated by 1.5-fold with CCA (Fig. 2C). COX activity was not significantly altered in response to mTORC1 inhibition in control cells (Fig. 2A), but yet COX IV and Tfam protein expression was significantly enhanced in rapamycin-treated control cells by 2- to 2.5-fold ($P < 0.05$; Fig. 2, B and C). Neither COX activity nor COX IV protein levels were significantly increased beyond the effect induced by CCA in the presence of rapamycin (Fig. 2, A and B). In contrast, a further increase in Tfam protein levels, by 1.5-fold, was also produced by rapamycin treatment that was greater than that observed with CCA alone (Fig. 2C).

To determine whether the observed changes in mitochondrial content were functional, we examined respiration in isolated mitochondria from all experimental conditions in the presence of glutamate (state 4), or with glutamate and ADP (state 3). There was no significant difference observed in state 4 respiration across the conditions (Fig. 3A). However, upon addition of ADP, state 3 respiration was significantly decreased by 33% with mTORC1 inhibition in control cells (Fig. 3B). CCA did not alter rates of state 3 respiration, but was effective in attenuating the observed decline in the presence of mTORC1 inhibition (Fig. 3B).

Expression of mTORC1-associated proteins. Important components involved in mTORC1 activity include the mTOR-associated protein raptor, phosphorylated mTOR (Ser²⁴⁴⁸), the transcription factor YY1, and the transcriptional coactivator PGC-1 α . Raptor protein levels were unaffected by CCA but were significantly increased in the presence of rapamycin (Fig. 4A). Levels of phosphorylated mTOR on Ser²⁴⁴⁸ were reduced by 26% with CCA in contrast with control cells (Fig. 4B). Rapamycin also reduced mTOR phosphorylation by 32%, but this was not further decreased in the presence of CCA (Fig. 4B). YY1 protein expression was also not influenced by CCA but was increased by 1.8- to 2.7-fold in the presence of rapamycin (Fig. 5A). As expected, CCA increased PGC-1 α protein expression by 1.3- to 1.5-fold; however, rapamycin, either in the presence or absence of CCA, had no effect on PGC-1 α protein levels (Fig. 5B).

Additional regulators of mTORC1 activity. mTORC1 is regulated by numerous signaling cascades, and a main kinase responsible for its deactivation is AMPK (17). Thus we evaluated the levels of AMPK under the influence of mTORC1 inhibition. Incubation of cells with rapamycin for 4 days had no effect on the expression of AMPK or on its phosphorylation at Thr¹⁷² (Fig. 6A). Prolonged inhibition of mTORC1 with rapamycin has also been demonstrated to impact the other mTOR complex, mTORC2 (34). Therefore, we examined the expression and phosphorylation status of Akt, a downstream target of mTORC2. Our results indicate that rapamycin treat-

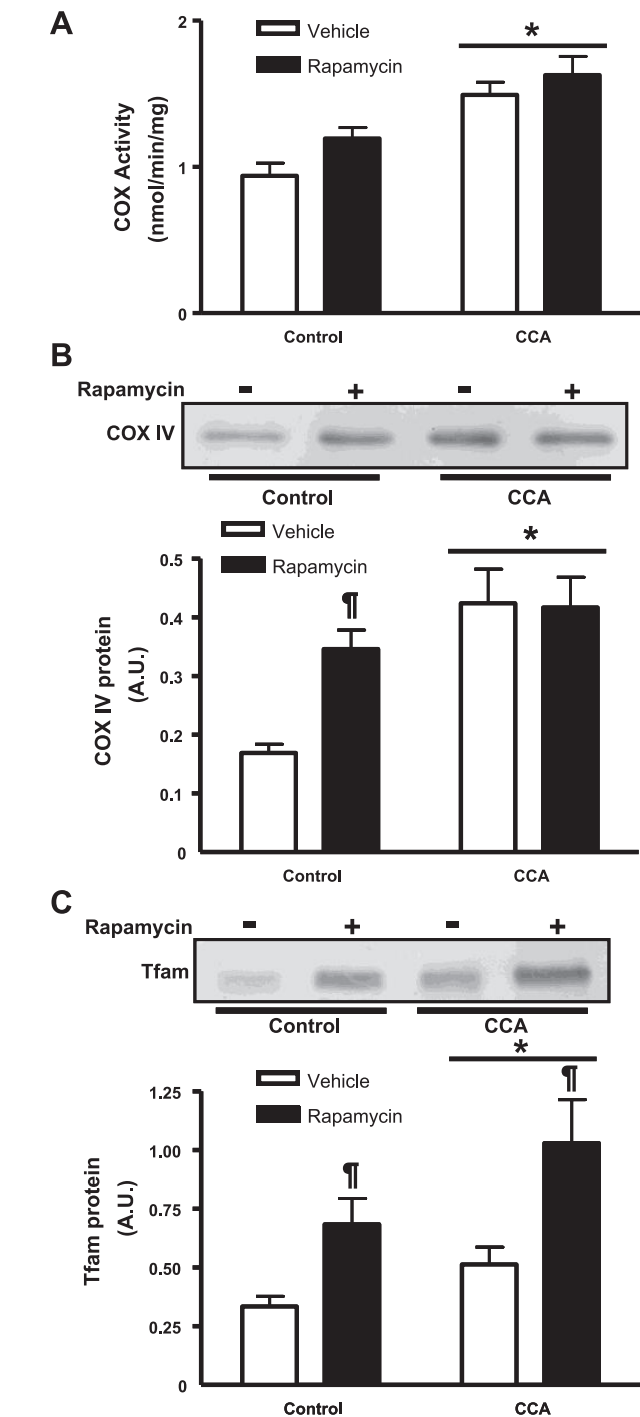


Fig. 2. CCA-induced mitochondrial biogenesis in the presence of rapamycin. Fully differentiated myotubes were chronically stimulated to induced mitochondrial biogenesis in the presence of vehicle or 1 μ M rapamycin. A: cytochrome oxidase (COX) activity ($*P < 0.05$, CCA vs. control; $n = 5$). B: COX IV protein expression ($\text{¶}P < 0.05$, rapamycin vs. vehicle; $*P < 0.05$, CCA vs. control; $n = 10$ experiments). C: Tfam protein expression ($*P < 0.05$, CCA vs. control; $\text{¶}P < 0.05$ rapamycin vs. vehicle; $n = 6$ experiments). AU, arbitrary units.

ment resulted in a 33% reduction of Akt phosphorylation (Ser⁴⁷³) after the duration of the protocol (Fig. 6B).

Acute effects of contractile activity on mTORC1. To determine the individual effects of one bout of contractile activity

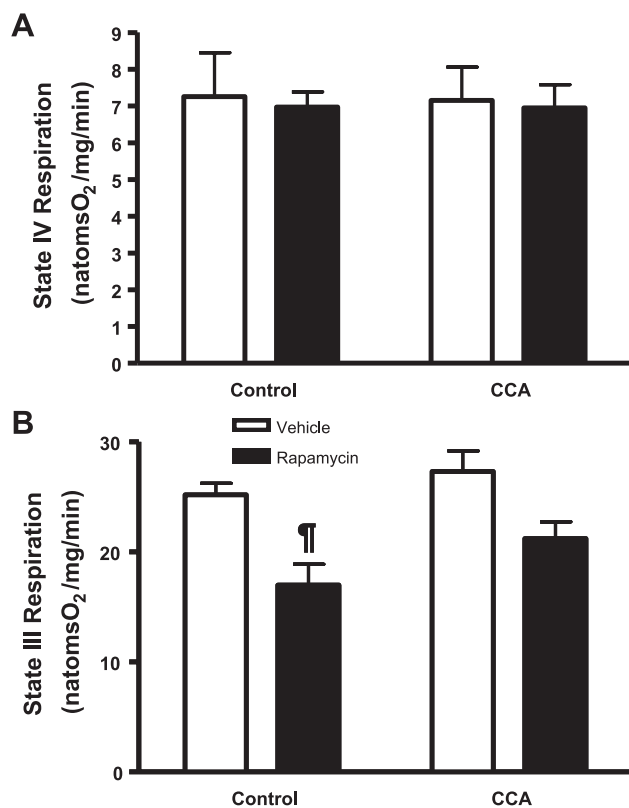


Fig. 3. Mitochondrial respiration. Following 4 days of CCA in the presence or absence of the mTORC1 inhibitor rapamycin, mitochondria were isolated by differential centrifugation and state 4 and state 3 respiration were measured. *A*: state 4 respiration [not significant (ns); $n = 4$ experiments]. *B*: state 3 respiration ($\#P < 0.05$, rapamycin control vs. vehicle control; $n = 4$ experiments).

on components of the mTORC1 complex and associated proteins, we exposed cells to an acute contractile activity protocol. Myotubes were stimulated for 2 h and allowed to recover for another 2 h. AMPK Thr¹⁷² phosphorylation tended to increase with contractile activity and was significantly reduced following the recovery period compared with acute contractile activity (Fig. 7*A*). We also evaluated raptor phosphorylation on Ser⁷⁹², an AMPK-mediated inhibitory phosphorylation. Raptor phosphorylation was upregulated by 1.5-fold ($P < 0.05$) with contractile activity, which returned to control levels during the recovery period (Fig. 7*B*).

In addition, we divided the myotubes into cytoplasmic and nuclear fractions to delineate the possible effects of acute contractile activity on protein translocation. YY1 was found exclusively in the nuclear fraction and was unaltered by contractile activity (Fig. 8*A*). Ser²⁴⁴⁸ phosphorylation of mTOR was unchanged within the cytoplasmic compartment (Fig. 8*B*), yet significantly increased in the nucleus during the recovery period (Fig. 8*C*). Total raptor levels were unchanged in both fractions (data not shown), and phosphorylated raptor on Ser⁷⁹² also remained unchanged in the cytoplasm (Fig. 8*D*). Within the nucleus, raptor phosphorylation was significantly increased with contractile activity, and this effect persisted during the recovery period (Fig. 8*E*).

DISCUSSION

mTORC1 is a complex of proteins that is established to play a role in many cellular processes including protein synthesis

and autophagy (37). Recently, mTORC1 has been demonstrated to participate in a transcriptional complex that promotes the expression of NUGEMPs in a model of quiescent muscle cells (9). Acute inhibition of mTORC1 with rapamycin led to a decrease in the expression of numerous nuclear-encoded mitochondrial mRNAs and mtDNA, while also manifesting a decline in mitochondrial function, as evaluated by oxygen consumption (9). This suggests that mTORC1 activity is required for the proper maintenance of mitochondrial content and function in muscle cells. However, it is unknown whether the activity of mTORC1 is required for changes in mitochondrial content with exercise. Additionally, how components of this transcriptional complex are regulated during the exercise-induced increase in mitochondrial content has not been investigated. Therefore, the purposes of our investigation were 1) to assess if mTORC1 activity is required for CCA-induced changes in mitochondrial content and 2) to evaluate the addi-

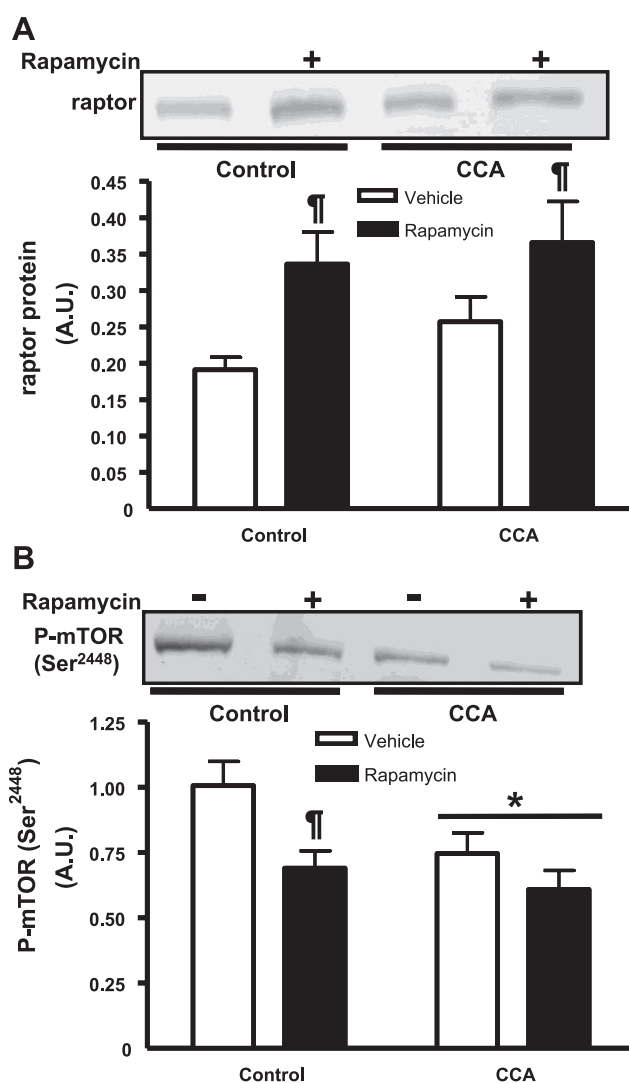


Fig. 4. Protein expression of integral mTORC1 components. Myotubes exposed to chronic stimulation and mTORC1 inhibition were collected and analyzed for protein content of raptor and P-mTOR Ser²⁴⁴⁸. *A*: raptor protein expression ($\#P < 0.05$, rapamycin vs. vehicle; $n = 10$ experiments). *B*: phosphorylation of mTOR on Ser²⁴⁴⁸ ($*P < 0.05$, CCA vs. control; $\#P < 0.05$, rapamycin control vs. vehicle control; $n = 11$ experiments). AU, arbitrary units.

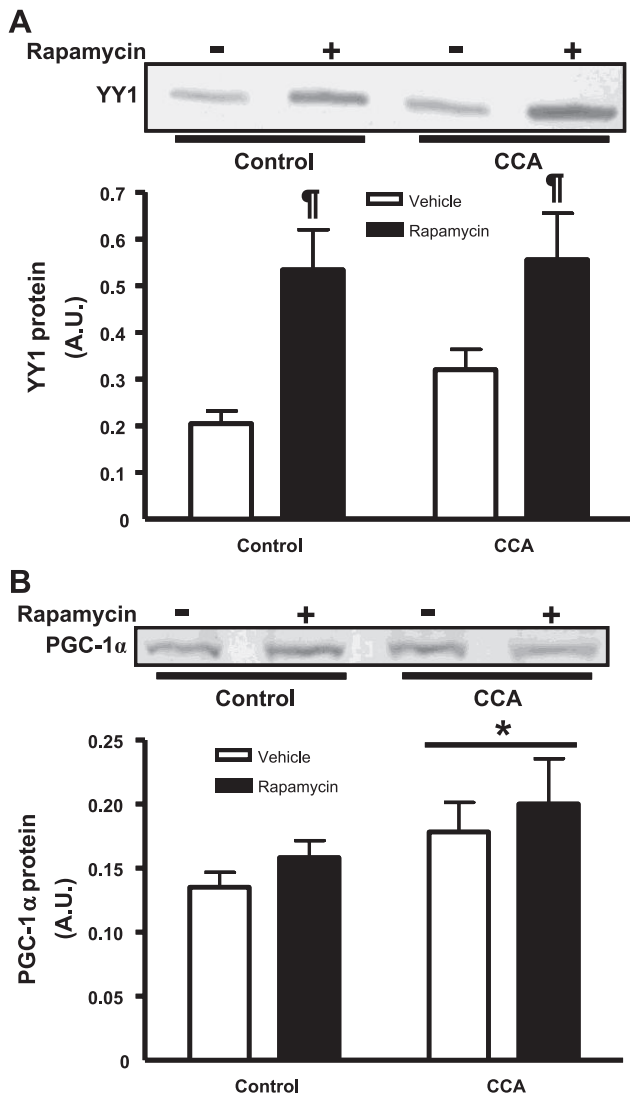


Fig. 5. Transcriptional associates of mTORC1. Protein levels of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and Yin Yang 1 (YY1) were analyzed after samples were harvested following 4-day CCA and mTORC1 inhibition. *A*: YY1 protein expression ($\#P < 0.05$, rapamycin vs. vehicle; $n = 7$ experiments). *B*: PGC-1α protein expression ($*P < 0.05$, CCA vs. control; $n = 4$). AU, arbitrary units.

tional components of the transcriptional complex, and how they respond to both acute and chronic contractile activity.

To determine whether mTORC1 activity was compulsory for CCA-induced mitochondrial biogenesis, we treated mature skeletal muscle myotubes with rapamycin to inactivate mTORC1, and subsequently induced CCA via electrical stimulation of the cells for 3 h per day, over 4 successive days. We have successfully used this paradigm to induce mitochondrial biogenesis for several years (8, 42). In the current study, we provide evidence of a CCA-induced increase in mitochondrial biogenesis because of the 1.6-fold increase in COX activity, as well as in the 2.5-fold increase in expression of COX subunit IV and the 1.5-fold increase in Tfam. These CCA-induced increases were completely unaffected by the presence of rapamycin, despite the dose employed (1 μM) and the clear effectiveness of the drug in inhibiting mTORC1 activity. Thus our observations suggest that mTORC1 activity was not re-

quired for the induction of mitochondrial biogenesis elicited by CCA.

Our results also indicate that inhibition of mTORC1 for 4 days may result in an upregulation of the expression of NUGEMPs in quiescent myotubes, including COX IV and Tfam. These data are reminiscent of observations that have been made in lower organisms, such as *S. Cerevisiae* (27, 28). Pan and Shadel (27) found that subunits of the electron transport chain increased with rapamycin treatment. However, this contrasts with the results of Cunningham et al. (9) who reported that a number of nuclear-encoded mitochondrial transcripts were suppressed by acute, short-term (14 h) rapamycin treatment. To evaluate this difference, we also treated resting myotubes with low (20 nM) and higher (1 μM) doses of rapamycin for 14 h and assessed the transcript levels of PGC-1α. We observed a reduction in PGC-1α mRNA levels with both concentrations of rapamycin with 14 h of treatment (data not shown). However, this effect appears to be short-lived, since our data indicate that PGC-1α protein levels were unaffected by more prolonged mTORC1 inhibition. Thus, the effect of mTORC1 inhibition on the expression of NUGEMPs in muscle appears to be time dependent. From a functional perspective, analysis of maximal, ADP-stimulated respiration rates revealed a significant reduction in quiescent myotubes, similar to that reported previously (9, 32, 35). Thus, despite the transient and divergent nature of the gene expression pattern evident with mTORC1 inhibition, the end result appears to be

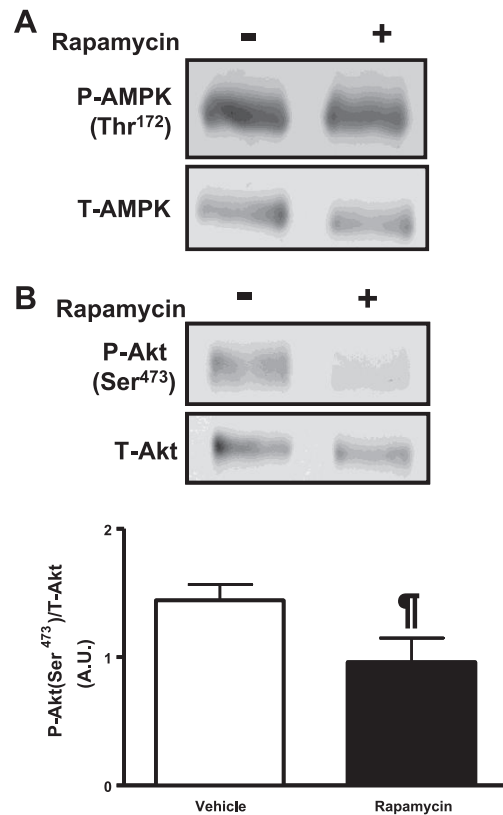


Fig. 6. Signaling of AMPK and Akt with chronic mTORC1 inhibition. To determine whether 5 days of rapamycin treatment altered other important signaling kinases associated with mTOR, the phosphorylation of AMPK and Akt was assessed. *A*: P-AMPK Thr¹⁷² and T-AMPK representative blots. *B*: P-Akt Ser⁴⁷³ ($\#P < 0.05$ rapamycin vs. vehicle; $n = 6$ experiments). AU, arbitrary units.

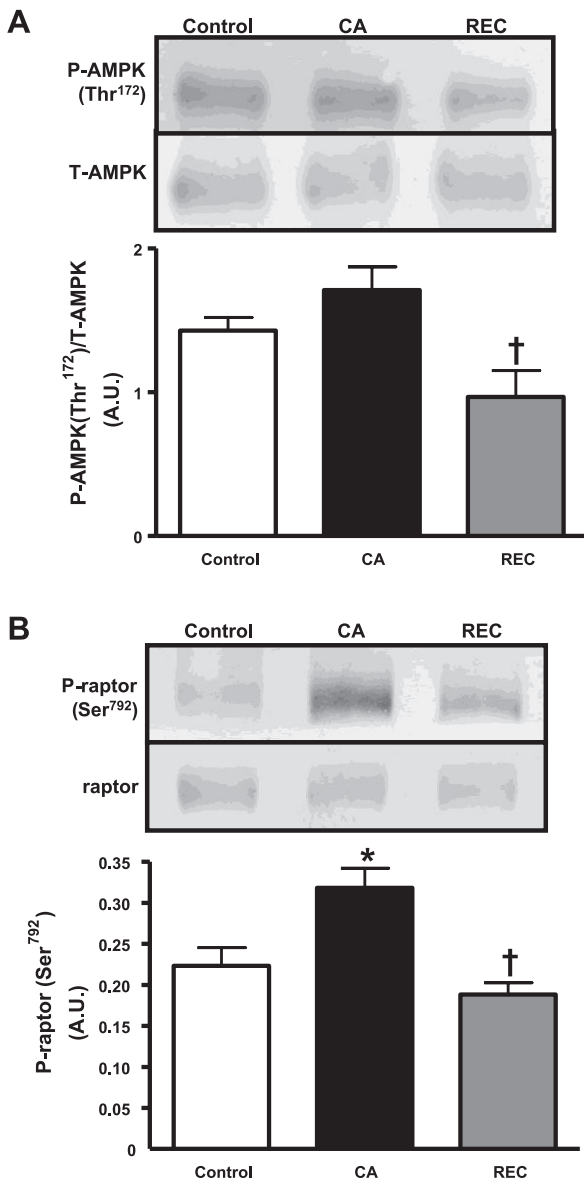


Fig. 7. Acute contractile activity (CA) effects on mTORC1. To investigate the immediate effects of contractile activity on mTORC1 signaling we subjected cells to 2 h of stimulation or allowed the myotubes 2 h of recovery following contractile activity (REC). **A:** P-AMPK Thr¹⁷² ($\dagger P < 0.05$, REC vs. CA; $n = 3$ experiments). **B:** raptor Ser⁷⁹² phosphorylation levels ($*P < 0.05$, CA vs. control; $\dagger P < 0.05$, REC vs. CA; $n = 6$ experiments). AU, arbitrary units.

dysfunctional mitochondria. This is not likely due to an impaired clearance of debilitated mitochondria, since the inhibition of mTORC1 should accelerate the autophagic degradation of poor quality mitochondria (i.e., mitophagy) (14, 44). It is more likely due to the altered regulation of NUGEMPs that are downstream of mTORC1 activity. Of course, this mechanism would only control a fraction of the total complement of mitochondrial proteins, thereby leading to altered organelle composition, and possible dysfunction. As is often the case in the existence of dysfunctional mitochondria, such as in mitochondrial disease, a compensatory upregulation of mitochondrial content can be evident (21, 39, 46), as our data suggest.

To fulfill our second purpose, we analyzed the effects of contractile activity on the components identified to be required

for the mTORC1 transcriptional complex, including PGC-1 α , raptor, phospho-mTOR (Ser²⁴⁴⁸), and YY1. Overall, CCA did not alter raptor or YY1 protein levels, whereas PGC-1 α increased as expected (42), as it does in exercising muscle (3, 20). This suggests that CCA works to increase mitochondrial content through mechanisms that do not include changes in YY1 or raptor expression. Others have also observed that neither exercise training nor static stretch alter the expression or DNA binding of YY1, respectively, in muscle (7, 31). However, it is currently unknown whether post-translational

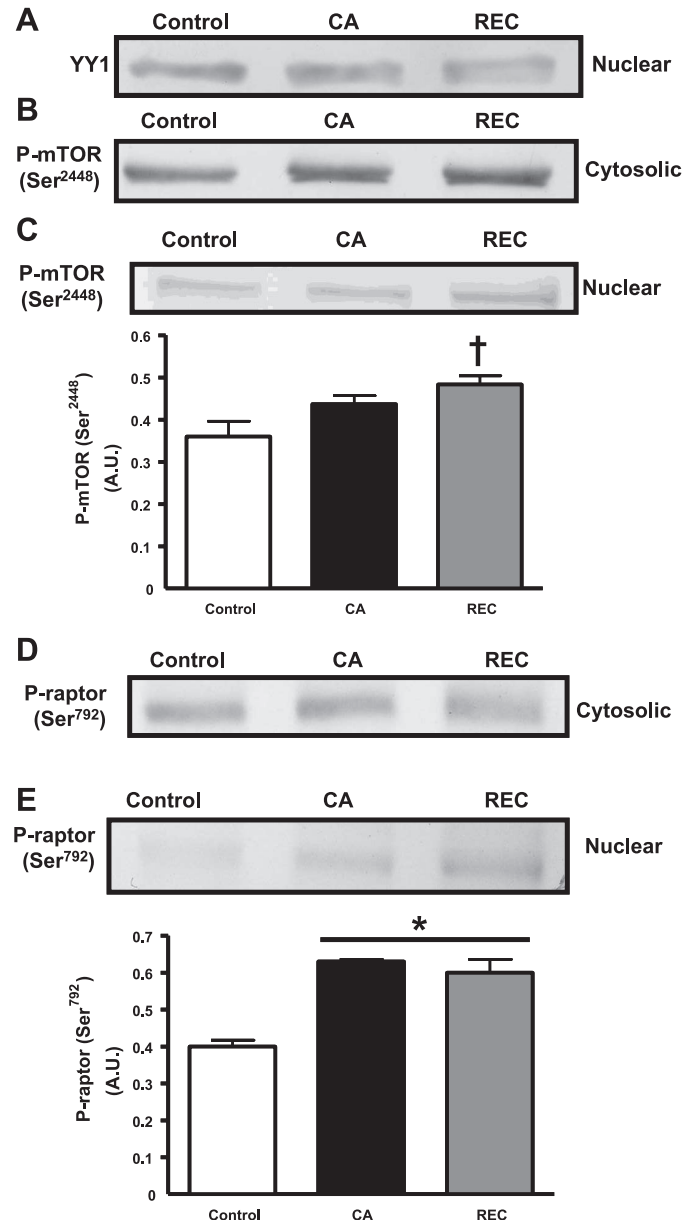


Fig. 8. Cytosolic and nuclear distribution of mTORC1 proteins and YY1. To examine whether contractile activity causes the translocation of mTORC1 components, or YY1, in myotubes, we used an acute bout of contractile activity followed by cellular fractionation into the cytosolic and nuclear compartments. **A:** nuclear YY1 protein expression. **B:** cytosolic phosphorylation of mTOR Ser²⁴⁴⁸. **C:** nuclear P-mTOR Ser²⁴⁴⁸ ($\dagger P < 0.05$, REC vs. control; $n = 3$ experiments). **D:** cytosolic P-raptor Ser⁷⁹². **E:** nuclear P-raptor Ser⁷⁹² ($*P < 0.05$, CA and REC vs. control; $n = 3$ experiments). AU, arbitrary units.

modifications of YY1, brought about by CCA, alter YY1 transcriptional activity.

With regard to raptor, studies have described that it can be phosphorylated on numerous serine residues, and these post-translational modifications can have a largely inhibitory impact on mTORC1 function (13, 17, 45). Indeed, activated AMPK has been identified to phosphorylate raptor, leading to mTORC1 inhibition, whereas the lack of AMPK in muscle prevents raptor Ser⁷⁹² phosphorylation (17, 22). Previous evidence has demonstrated that AMPK phosphorylation is increased by contractile activity in vivo (24) and with the current CCA model (42). Thus we speculate that CCA would upregulate raptor phosphorylation and reduce mTORC1 activity, while maintaining the CCA-induced increase in mitochondrial biogenesis via mechanisms that are independent of mTORC1 activity. In support of this, AMPK Thr¹⁷² phosphorylation tended to increase, while AMPK-mediated (17, 22) raptor Ser⁷⁹² phosphorylation was significantly augmented after a 2-h bout of contractile activity. These results suggest that acute contractile activity mediates AMPK-induced raptor phosphorylation, which could contribute to the transient attenuation of mTORC1 activity during each bout of contractile activity. This negative influence on mTORC1 activity likely occurs within the nucleus, since we observed that the abundance of phosphorylated raptor increased in the nuclear fraction during contractile activity and also remained elevated during the recovery period.

In contrast, numerous studies have indicated that acute exercise in humans and in rodents results in the phosphorylation of mTOR, leading to the activation of downstream targets (12, 26, 29). This is particularly evident with resistance, high intensity exercise, or with synergist ablation (5, 10, 11). Our results also reflect this, since an acute bout of contractile activity led to an increase in mTOR phosphorylation within the nucleus. However, the time-dependent nature of this activation becomes evident during the recovery phase, whereby a marked reduction in mTOR phosphorylation was found following 21 h of recovery. This occurrence has also been documented in animals subjected to 9 wk of voluntary wheel running, with the tissues being collected 24 h after the last opportunity for exercise (16). This may be due to a rebound reduction in kinase activation that is often evident during a prolonged recovery period. For example, following endurance exercise, the phosphorylation of Akt is immediately increased, but subsequently becomes markedly reduced by 5 h into the recovery phase (43). We also noted this time-dependent phenomenon in other studies (23), and in the current investigation with respect to AMPK phosphorylation 2 h post-contractile activity. In addition, kinase activation is also typically reduced in response to contractile activity if mitochondrial content is elevated (24) within the muscle. A combination of these time- and adaptation-dependent events likely accounts for the decreased mTORC1 phosphorylation following 4 days of contractile activity, accompanied by a recovery period.

We also hypothesized that the inhibition of mTORC1 would impair mitochondrial biogenesis as suggested by Cunningham et al. (9). Thus we utilized the long-standing mTORC1 inhibitor, rapamycin, under control noncontracting conditions to evaluate this. Inhibition of mTORC1 led to an increase in raptor and YY1, as well as the mitochondrial marker COX subunit IV, following 4 days of treatment. Thus these data

suggest that mTORC1 activity negatively regulates specific components of the mTORC1 transcriptional complex, and when this repression is removed, an upregulation of these proteins is evident, accompanied by the increased expression of mitochondrial markers. This occurred in the absence of a change in PGC-1 α protein levels, which likely contributed to the maintenance of mitochondrial content in the presence of mTORC1 inhibition. In contrast, the decline in mitochondria observed within muscle of animals subject to genetic ablation of either mTOR or raptor (4, 32, 33) is likely due to the accompanying decrease in PGC-1 α , as measured at the mRNA level.

In summary, our data suggest that CCA-induced mitochondrial biogenesis within skeletal muscle is not reliant on mTORC1, since inhibition of this protein complex did not perturb the increase in mitochondrial markers observed with CCA. Furthermore, prolonged mTORC1 inactivation in quiescent myotubes led to the upregulation of certain mitochondrial indicators, although this came at the expense of mitochondrial respiration, reminiscent of cells with mitochondrial disease. This defect could be recovered with the addition of CCA, further fortifying a role for contractile activity in ameliorating mitochondrial dysfunction in muscle (2, 21).

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: H.N.C. performed experiments; H.N.C. analyzed data; H.N.C. and D.A.H. interpreted results of experiments; H.N.C. prepared figures; H.N.C. drafted manuscript; H.N.C. and D.A.H. edited and revised manuscript; D.A.H. conception and design of research; D.A.H. approved final version of manuscript.

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