Influence of aerobic exercise intensity on myofibrillar and mitochondrial protein synthesis in young men during early and late post-exercise recovery

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Running head: Aerobic exercise and muscle protein synthesis

Article words count (exclusive of references and legends): 4479

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ABSTRACT

Aerobic exercise is typically associated with expansion of the mitochondrial protein pool and improvements in muscle oxidative capacity. The impact of aerobic exercise intensity on the synthesis of specific skeletal muscle protein sub-fractions is not known. We aimed to study the effect of aerobic exercise intensity on rates of myofibrillar (MyoPS) and mitochondrial (MitoPS) protein synthesis over an early (0.5-4.5 h) and late (24-28 h) period during post-exercise. Using a within subject crossover design, eight males (21 ± 1 years, VO2 peak: 46.7 ± 2.0 mL•kg⁻¹•min⁻¹) performed two work-matched cycle ergometry exercise trials (LOW: 60 min at 30% Wmax; HIGH: 30 min at 60% Wmax) in the fasted state while undergoing a primed constant infusion of L-[ring-¹³C₆]phenylalanine. Muscle biopsies were obtained at rest, and 0.5, 4.5, 24, and 28 h post-exercise to determine both the ‘early’ and ‘late’ response of MyoPS and MitoPS and the phosphorylation status of select proteins within both the Akt/mTOR and MAPK pathways. Over 24-28 h post-exercise, MitoPS was significantly greater after the HIGH vs. LOW exercise trial (P < 0.05). Rates of MyoPS were increased equivalently over 0.5-4.5 h post-exercise recovery (P < 0.05), but remained elevated at 24-28 h post-exercise only following the HIGH trial. In conclusion, an acute bout of high, but not low intensity aerobic exercise in the fasted state resulted in a sustained elevation of both MitoPS and MyoPS at 24-28 h post-exercise recovery.

Abbreviations: Akt, protein kinase B; COXIV, cyclooxygenase IV; ERK, extracellular signal-regulated kinase; FSR, fractional synthetic rate; H2B, histone 2B; HIGH, high intensity; LDH, lactate dehydrogenase; LOW, low intensity; MAPK, mitogen-activated protein kinase; MHCI, myosin heavy chain I; MitoPS, mitochondrial protein synthesis; MyoPS, myofibrillar protein synthesis, mTOR, mammalian target of rapamycin; p70S6K1, 70kDa S6 protein kinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.
INTRODUCTION

Adaptations to aerobic-based exercise include increases in mitochondrial protein content (both size and number of mitochondria) and subsequent improvements in muscle oxidative capacity and resistance to fatigue (20). Additionally, traditional aerobic exercise (16, 18) as well as high intensity “sprint” training (19, 33) can also enhance skeletal muscle hypertrophy, an adaptation that would be contingent upon stimulation of myofibrillar protein synthesis (MyoPS) and expansion of the myofibrillar protein pool (31). Specific phenotypic outcomes (i.e. improved oxidative capacity and muscle hypertrophy) in response to divergent exercise stimuli must relate to changes in the synthesis of specific muscle protein sub-fractions and may be altered by the intensity of exercise (35). For example, work-matched performance of high intensity resistance exercise results in greater rates of MyoPS than low intensity resistance exercise (6, 22). An increase in the rate of mixed muscle protein synthesis has been reported after an acute bout of aerobic based exercise (8, 17, 27); however, such measures preclude insight into the synthetic response of specific muscle protein sub-fractions including mitochondrial and myofibrillar proteins. Whether manipulation of aerobic exercise intensity alters the synthesis of specific muscle protein sub-fractions is unknown.

Phosphorylation resulting in activation/deactivation of proteins in the Akt-mTOR pathway has been shown to be critical in the regulation of contraction-mediated increases in protein synthesis (12). Other contraction dependent signaling pathways, such as the MAPK pathway, may also be involved in regulating contraction-mediated translational control (34, 36). Phosphorylation of p38 MAPK can affect transcription factors such as peroxisome proliferator-activated receptor 1 coactivator α (PGC-1α) (25, 34, 36). As a primary regulator of mitochondrial biogenesis, PGC-1α coordinates transcriptional activity and assists in coordinating the transcription of mitochondrial and nuclear DNA for mitochondrial biogenesis (13, 30). Higher intensity aerobic exercise has been demonstrated to result in a
greater increase in the mRNA abundance of PGC-1α when compared with lower-intensity exercise (13), but is unknown whether aerobic exercise intensity alters rates of mitochondrial protein synthesis (MitoPS).

The purpose of the present study was to examine the effect of acute bouts of work-matched aerobic exercise of different intensities on rates of MyoPS, MitoPS, and the phosphorylation status of signaling molecules of the Akt-mTOR and MAPK pathways during early (4 h) and late (24 h) post-exercise recovery. To examine the independent effects of exercise, we chose to study subjects in the fasted state. We hypothesized that high intensity (HIGH) cycle ergometry would elicit greater increases in MyoPS and MitoPS than work-matched low intensity (LOW) cycling exercise. Additionally, we hypothesized that signaling molecule phosphorylation would align with the intensity-dependent differences in protein synthesis with greater activation of the Akt/mTOR and MAPK pathways and increased nuclear PGC-1α accumulation following HIGH exercise.

METHODS

Participants. Eight healthy, recreationally active men (mean ± SEM, 21 ± 1 years, 82.5 ± 3.8 kg, 181 ± 2 cm, VO₂ peak: 46.7 ± 2.0 mL kg⁻¹ min⁻¹) were recruited to participate in the study. Participants reported participating in unstructured moderate intensity aerobic exercise 1-2 times per week. All participants were informed of the purpose of the study, experimental procedures and associated risks prior to participation and exercise testing. All participants gave verbal and written consent to a protocol approved by the Hamilton Health Science Research Ethics Board, conforming to the standards for the use of human subjects in research as, outlined in the Declaration of Helsinki and with current Canadian Tri-council research agency guidelines for use of human participants in research (http://www.pre.ethics.gc.ca/eng/policy-politique/initiatives/tcps2-epc2/Default/).
Experimental Design. The study consisted of pre-study maximal aerobic capacity measures, a brief familiarization session to assess the aerobic exercise intensities for the trials, and finally two infusion trials for resting and post-exercise metabolic investigation per exercise intensity (4 infusion trials total). Each participant completed both exercise intensity trials making this study a within-subject crossover design.

Maximal aerobic capacity measurements. Two weeks prior to the first infusion trial, participants reported to the laboratory and completed a VO₂ peak test on a cycle ergometer (Lode, Groningen, Netherlands) with continuous oxygen uptake measurements (Ergocard Professional, Medisoft, Sorinnes, Belgium). The test began at 50 W and increased 1 W every 2 s until voluntary fatigue. Fatigue was defined by a respiratory exchange ratio greater than 1.1 and the inability to maintain 60 revolutions per minute (rpm) on the cycle ergometer despite vigorous verbal encouragement. Peak power output in Watts (W_{max}), maximum heart rate (HR_{max}), and average cadence were recorded. Participants were asked to maintain a constant cadence (within 5-10 rpm) between 70 and 100 rpm. The position of the saddle and handlebars was recorded for each participant and was repositioned accordingly for each subsequent exercise bout. The W_{max} for each participant was used to determine the workload for the relative high (HIGH, 60% W_{max}) and low intensity (LOW, 30% W_{max}) exercise trials.

Familiarization trial. A familiarization session (~15 min) was carried out with each participant with the exercise intensity that was performed on the days of metabolic investigation, and also to confirm the relative intensity of the exercise based on HR and VO₂. One week prior to the first infusion trial, participants completed a short bout of exercise at LOW and then HIGH workloads. Heart rate was measured throughout the familiarization and oxygen consumption was also measured in the last 2 min of both HIGH and LOW. The participants were asked to maintain the same constant pedaling cadence that was comfortable
for them during the maximum aerobic capacity test, which was also maintained during exercise trials.

Metabolic investigation and infusion protocol. Participants underwent two experimental infusions on sequential days for both HIGH and LOW exercise (4 trials total) to study the response of both MyoPS and MitoPS during early (0.5-4.5 h) and later (24-28 h) post-exercise recovery. At least 10 days separated the trials for the two intensities. Participants were asked to keep a diet record for the 48 h period preceding the first infusion protocol corresponding to each of the exercise trials (Day 1 and Day 3 respectively). A standardized meal representing 30% of each subject’s energy requirements (64% CHO, 17% PRO, 19% fat) was provided and consumed by 2000 h on the evening before the first infusion trial for each condition. After an overnight fast and after refraining from physical activity for 2 days prior to the trial, participants reported to the laboratory at 0600 h for Day 1 (Figure 1). A 20 gauge catheter was inserted into an antecubital vein of one arm and a baseline blood sample was obtained. The catheter was kept patent with 0.9% saline drip for repeated blood sampling. A second catheter was then inserted into the other arm for a primed constant infusion of L-[ring-$^{13}$C$_6$]phenylalanine (prime: 2 μmol kg$^{-1}$; infusion: 0.05 μmol kg$^{-1}$; Cambridge Isotope Laboratories, Cambridge, MA, USA) which passed through a 0.2 μm filter. Participants rested on a bed until 3 h into the infusion at which point a biopsy (~100-150 mg) was obtained from the vastus lateralis for fasted resting measurements. Muscle biopsies were obtained under local anesthesia (2% xylocaine) using a 5 mm Bergström needle modified for manual suction. Tissue obtained was blotted, freed of any visible connective tissue and fat, and immediately frozen in liquid N$_2$ and stored at -80 °C until analysis. After the resting biopsy, participants began the exercise protocol on the same cycle ergometer that was used in the VO$_2$ peak test. Participants were randomized to complete the HIGH or LOW intensity protocol during their first trial. The HIGH protocol consisted of 30 min at 60% $W_{\text{max}}$ and the LOW protocol
consisted of 60 min at 30% $W_{\text{max}}$. By work-matching the protocols, we aimed to remove the influence of total energy expenditure during exercise as a potential confounding variable. Energy expenditure did not differ between the two exercise trials (Table 1). Measurements of HR were taken throughout the exercise bout, and VO$_2$ measurements were obtained three times during each ride. The participants returned to a bed to rest until 30 minutes after exercise at which point the second biopsy was obtained. After another 4 h of tracer infusion, a third biopsy was obtained and then the infusion was terminated. The diet was standardized for the infusion and subsequent day by providing participants with a meal immediately after the trial representing 50% of their daily caloric requirements and then providing a meal of identical macronutrient and caloric composition to consume in the evening before 2200 h. The next day participants returned to the laboratory at 0700 h after an overnight fast to undergo a second infusion (Day 2; Figure 1). The infusion protocol was carried out as in Day 1, with biopsies obtained at 1.5 h and 5.5 h into the infusion to obtain 24 – 28 h post-exercise measurements. Approximately 2 weeks later, the participants returned to the laboratory to complete Day 3 and Day 4 (Figure 1) performing the opposite exercise intensity to their first trial. The first biopsy was obtained 2 hours into the infusion on both days, with exercise beginning at the appropriate time so that the biopsy was obtained 30 minutes post-exercise.

**Blood and Muscle Analysis.** All blood samples were collected in heparinized evacuated containers and kept on ice until they were centrifuged to obtain plasma which was subsequently aliquoted, frozen, and stored at -20 °C until further analysis. Plasma $[^{13}\text{C}_6]$phenylalanine enrichments were determined as previously described (6). Muscle intracellular (IC) free amino acids were extracted from a 10-15 mg piece of wet muscle with ice-cold 0.6 M perchloric acid (PCA) and purified as previously described (5, 6). Purified free amino acids were then converted to their heptafluorobutyrate (HFB) derivatives and analyzed
for $[\text{ring}^{-13}\text{C}_6]$-phenylalanine enrichment by a GC-MS (GC:6890, MS:5973, Hewlett-Packard, Palo Alto, CA, USA) as previously described (29).

A ~100 mg piece of wet muscle was homogenized using a glass homogenizer in ice-cold homogenization buffer (10 $\mu$L • mg$^{-1}$; 0.067 M sucrose, 0.05 M Tris/HCl, 0.05 M KCl, 0.01 M EDTA) with protease and phosphatase inhibitor cocktail tablets (Complete Mini, PhosSTOP, Roche Applied Science, Mannheim, Germany). The homogenate was transferred to an Eppendorf tube and centrifuged at 700 g for 15 min at 4 °C to pellet myofibrillar proteins. The supernatant was transferred to another Eppendorf tube and centrifuged at 12000 g for 20 min at 4 °C to pellet mitochondria. Both the extract and the supernatant were frozen at -80 °C until further analysis.

Amino acids were obtained from the mitochondrial pellet as described previously (4-6). Briefly, the pellet was washed twice with ice-cold homogenization buffer, once with ethanol and then dried under vacuum. Proteins were hydrolyzed by adding 6 N HCl and heating at 110 °C for 18 h. From the myofibrillar enriched pellet, nuclear proteins were extracted. The myofibrillar-enriched pellet was washed with ice-cold homogenization buffer and centrifuged at 700x g for 10 min at 4 °C. Three times, the pellet was washed with ice-cold PBS containing protease and phosphatase inhibitors and centrifuged at 15,000 g for 5 min at 4 °C. The pellet was fully resuspended in 4 $\mu$L of high salt buffer (HSB; 0.05 M Tris/HCl, 0.4 M NaCl, 0.001 M DTT, 0.001 M EGTA, 0.001 M EDTA, 0.1% SDS; added protease and phosphatase inhibitors) for every 1 mg of original wet tissue weight. The resuspended pellet was incubated on ice for 20 min, and was vortexed twice throughout. The Eppendorf tube was then placed in a sonication bath for 20 min at 4 °C followed by vortexing. The resuspended pellet was again incubated on ice for 20 min, vortexing every 10 min and then was centrifuged at 15,000 g for 10 min at 4 °C. The resulting supernatant (nuclear extract) was
transferred to an Eppendorf tube and a 100 μL 1/10 dilution was made for use in a BCA assay. Both the extract and the diluted supernatant were frozen at -80 °C until further analysis.

The myofibrillar enriched pellet was washed with H2O and centrifuged at 15,000 g for 5 min at 4 °C. Myofibrillar proteins were further extracted and hydrolyzed as described previously (4-6). The free amino acids from the mitochondrial and myofibrillar enriched fractions were purified using cation-exchange chromatography (Dowex 50WX8-200 resin, Sigma-Aldrich Ltd, St. Louis, MO, USA) and converted to their N-acetyl-n-propyl ester derivatives for gas chromatography combustion isotope ratio mass spectrometry (GC: 6890, Hewlett Packard, Palo Alto, CA, USA; IRMS: Delta Plus XP, Thermo Finnigan, Waltham, MA, USA). The enrichment of the myofibrillar and mitochondrial protein fractions using these methods were confirmed with Western blotting, using MHCI, COXIV and LDH as respective myofibrillar, mitochondrial, and sarcoplasmic markers, respectively. Using these methods, only MHCI was detectable in the myofibrillar protein fraction. In some samples (~15%), trace amounts of MHCI were present in the mitochondrial protein fraction; thus, the isolated mitochondrial fraction was highly enriched with COXIV proteins, and there was no detectable LDH (data not shown); we have previously confirmed the purity of these protein fractions (35).

**Immunoblot analysis.** Both sarcoplasmic and nuclear extracts were used for immunoblot analysis for presence and/or phosphorylation of signaling molecules. The protein concentration of the extracts was determined using the BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). Samples were prepared to the same concentration by dilution with distilled deionized H2O and denatured with Laemmli sample buffer and heated to 95 °C. On a 10% SDS-PAGE gel, 20-40 μg of protein (depending on the protein target) was loaded and ran at 120 V for 1-1.5 h. Proteins were transferred onto a PVDF membrane using Fast Semi-Dry Transfer (Thermo Fisher Scientific Inc., Rockford, IL, USA). Membranes were blocked
Membranes were incubated in primary antibody in TBST at 4 °C overnight: Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) rabbit polyclonal phospho-p70S6K1 (Thr389; 1:1,000 in TBST; #SC11759-R), Abcam (Cambridge, MA, USA) rabbit polyclonal histone 2B (0.1 μg/mL in TBST, #ab1790) and Cell Signaling Technology (Danvers, MA, USA) rabbit polyclonal phospho-mTOR (Ser2448; 1:1,000 in TBST, #2971), rabbit monoclonal phospho-p38 MAPK (Thr180/Tyr182; 1:1,000 in TBST; #4511), rabbit polyclonal phospho-ERK1/2 (Thr202/Tyr204; 1:1,1000 in TBST, #9101), and rabbit monoclonal alpha-tubulin (1:2,000 in TBST, #2125). Membranes were washed with TBST and then incubated with secondary anti-rabbit HRP-linked antibody (1:10,000 in TBST; GE Healthcare Life Sciences Ltd., Baie D’Urfe, QC, Canada; NA934) at RT for 1 h. After washing, membranes were visualized using chemiluminescence (Supersignal West Dura Extended Substrate, Thermo Fisher Scientific Inc., Rockford, IL, USA), and imaged using Fluorochem SP Imaging system (Protein Simple: Alpha Innotech, Santa Clara, CA, USA). Images were quantified using National Institute of Health ImageJ software, and normalized to the appropriate loading control. Alpha-tubulin and Histone 2B were used as loading controls in sarcoplasmic and nuclear protein samples respectively. Both α-tubulin and histone 2B were demonstrated to be valid loading controls (i.e., representative of total protein loaded, determined by quantifying ponceau staining) for sarcoplasmic and nuclear protein extracts, respectively. The degree of enrichment of sarcoplasmic and nuclear protein extracts was determined using Western blotting using MHC1, LDH, and COXIV as markers. Most important to our analysis, the sarcoplasmic fraction was free of detectable COXIV (mitochondrial proteins) and the nuclear extract was free of detectable LDH (sarcoplasmic proteins).

**Calculations.** The fractional synthetic rates (FSR) of myofibrillar and mitochondrial proteins were calculated using the precursor-product equation:
FSR (%/h) = \( \frac{(E_{2b} - E_{1b})}{E_p} \times \frac{1}{t} \times 100 \)

\( E_{2b} \) and \( E_{1b} \) are the bound protein enrichments at times 2 and 1 respectively and \( E_p \) is the average enrichment of the precursor, intracellular phenylalanine, during steady state. Since participants were ‘tracer naïve’, the baseline pre-infusion blood sample enrichment represents the naturally abundant \(^{13}\text{C} \) enrichments, and was used for \( E_{1b} \) to determine resting FSR. In this calculation we used an incorporation time from 30min after the start of the infusion to the time of the biopsy, which has been previously validated (3).

**Statistical Analysis.** Aerobic exercise trial data was analyzed using two-tailed paired sample Student’s \( t \)-test. Data within an exercise trial (%HR\(_{\text{max}}\), %VO\(_2\) peak, and plasma enrichment) were analyzed using a two-way repeated measures analysis of variance (ANOVA). To isolate differences between means for which there was not a resting value in each condition, immunoblot and FSR data were analyzed using a one-way analysis of variance (ANOVA), with structured contrasts to determine time- and condition-dependent differences. When appropriate, *post hoc* analysis was performed with a Student-Newman-Keuls test to isolate significant pair-wise differences. Correlations were 2-tailed Pearson correlations. All statistical analyses were performed using SPSS Statistics (Version 19, IBM, Armonk, NY, USA). All data are presented as means ± SEM. Statistical significance was accepted at \( P < 0.05 \).

**RESULTS**

**Aerobic Exercise Trial.** All participants completed the exercise at the prescribed intensity. The average %VO\(_2\) peak and %HR\(_{\text{max}}\) was significantly higher in the HIGH trial than in the LOW trial (%VO\(_2\) peak: 76 ± 3 vs. 48 ± 1; %HR\(_{\text{max}}\): 90 ± 1 vs. 66 ± 2; \( P < 0.001 \); Table 1).

During the HIGH trial, %VO\(_2\) peak was higher in the final 5 minutes of the bout than at 10 minutes into the exercise bout (\( P < 0.05 \)). The same was also observed for %HR\(_{\text{max}}\) during the
HIGH exercise trial ($P < 0.05$). No change in %$V_O^2_{peak}$ was observed during the LOW exercise bout. Total work was not different between HIGH and LOW trials (Table 1; $P = 0.46$).

**Plasma and intracellular enrichments.** The free plasma tracer enrichment was not different between the 0.5-4.5 and 24-28 h post-exercise incorporation times.

**Protein Synthesis.** Myofibrillar FSR was increased in early recovery (0.5-4.5 h) compared to rest in both exercise trials ($P < 0.05$; Figure 3A). In late recovery (24-28 h), myofibrillar FSR returned to rest in the LOW trial, but remained elevated with HIGH exercise ($P = 0.05$).

Mitochondrial FSR was significantly different between HIGH and LOW conditions in late recovery ($P < 0.05$; Figure 3B). Western blot images of MHCI (shown in green), LDH (shown in blue), and COXIV (shown in red) in the myofibrillar, sarcoplasmic, nuclear, and mitochondrial preparations are shown in Figure 6.

**Cell Signaling.** There was a significant difference at 0.5 h post exercise between the HIGH and LOW trials for phospho-mTOR$^{Ser2448}$ ($P < 0.005$; Figure 4A). This effect was no longer present at 4.5 h post-exercise. At 24 h and 28 h post-exercise, phospho-mTOR$^{Ser2448}$ was not different from resting levels in either condition. There was no effect of the exercise on phospho-p70S6K1$^{Thr389}$ at the time points measured (Figure 4B). Myofibrillar FSR in early and late recovery after HIGH exercise was positively correlated with phospho-mTOR$^{Ser2448}$ at 0.5 h post exercise ($r = 0.953$, $P < 0.001$ and $r = 0.866$, $P < 0.01$, respectively). No relationship was observed between phospho-p70S6K1$^{Thr389}$ and myofibrillar FSR (data not shown). Aerobic exercise did not result in an increase the phosphorylation of ERK1/2$^{Thr202/Tyr204}$ (Figure 4C). There was a main effect of time for the phosphorylation status of p38$^{Thr180/Tyr182}$ ($P < 0.005$; Figure 4D) where phosphorylation was significantly higher at 4.5 h post-exercise compared to rest, 0.5, 24, and 28 h post-exercise ($P < 0.05$). There was no
effect of aerobic exercise on nuclear PGC-1α content at the time points examined (Figure 4E).
Representative blot images are shown in Figure 5.

DISCUSSION

We report here that both high and low intensity aerobic exercise stimulate increases in MyoPS during early (0.5-4.5 h) recovery, whereas in late recovery (24-28 h), a sustained elevation in MyoPS was only observed in the HIGH trial (Figure 2A). We observed an increase in the phosphorylation status of mTOR\(^{\text{Ser2448}}\) only after the HIGH trial, the extent of which was correlated with rates of MyoPS during early and late recovery. This observation is interesting in light of the knowledge that aerobic exercise can serve as a stimulus to promote muscle hypertrophy under certain conditions (16, 18). Thus, it is possible that higher intensity aerobic exercise may, over time, induce a degree of muscle hypertrophy not seen with low intensity aerobic exercise. Alternatively, the sustained elevation in MyoPS rates at 24 h post-exercise following the HIGH trial may reflect an increase in muscle protein turnover to assist in the remodeling and protein renewal. Our findings demonstrate that aerobic exercise intensity influenced the synthesis of specific muscle protein fractions, which has bearing on the interpretation of findings from studies of mixed muscle protein synthesis following endurance exercise (17, 27). It has previously been reported that in the fed state, rates of MyoPS are increased above resting values 24 h after both resistance exercise (4, 6) and high-intensity aerobic exercise (11, 28). Thus, loading appears to ‘sensitize’ the muscle to protein provision late into the post-exercise recovery period, which also appears true even with low intensity endurance exercise (15). In support of this notion, we observed intensity-dependent differences in the phosphorylation of mTOR\(^{\text{Ser2448}}\) at 0.5 h following the HIGH, but not LOW exercise trial (Figure 3A). An increase in the phosphorylation of mTOR\(^{\text{Ser2448}}\) after cycling exercise has been observed previously (7, 9, 10, 26, 27, 35), but the response appears transient with aerobic as compared to resistance exercise (7). To our knowledge, ours is the first report
of an aerobic exercise intensity-dependent effect on mTOR\textsuperscript{Ser2448} phosphorylation. Interestingly, mTOR\textsuperscript{Ser2448} phosphorylation at 0.5 h was correlated to rates of MyoPS in early and late recovery from the HIGH exercise trial. However, the phosphorylation of p70S6K\textsuperscript{Thr389}, which is often taken as a proxy of mTOR activity and is a known regulator of mRNA translation initiation and elongation (21), was not elevated. This finding is in agreement with previous work for Mascher and colleagues (2007) who reported that a 1-hour bout of cycle ergometer exercise at \(~75\%\) VO\textsubscript{2max} increased the phosphorylation status of mTOR\textsuperscript{Ser2448} at 0.5 h, but had no effect on p70S6K\textsuperscript{Thr389} (26). The lack of change in p70S6K\textsuperscript{Thr389} phosphorylation also corroborates results from previous studies of aerobic exercise in which biopsies were taken at similar time points (7, 9, 10). It should however, be acknowledged that a single phosphorylation site (Ser 2448) of mTOR was measured; other phosphorylation sites that regulate mTOR activity were not measured. It is not clear what role, if any, the early divergent response of mTOR\textsuperscript{Ser2448} phosphorylation may have played in mediating the divergent protein synthesis responses observed in late recovery. More research is required to determine the mechanisms determining the sustained increases in MyoPS during late (e.g., 24 h) post-exercise recovery.

In the present study, aerobic exercise, irrespective of intensity, did not elicit a significant increase from rest in fasted-state measures of MitoPS during early or late recovery, although there was a trend for such a response (Figure 2B; \(P=0.18\)). We did however observe divergent responses in late recovery, whereby MitoPS rates were higher after the HIGH versus LOW exercise trial. These results are in contrast with our previous findings of a stimulation of MitoPS following aerobic exercise (35); however, our previous study was performed under conditions of sustained hyperinsulinemia and hyperaminoacidemia, which may explain the divergent findings. Others have reported that protein provision before high-intensity repeated sprinting (9) and protein plus carbohydrate ingestion after prolonged higher...
intensity aerobic exercise (2) did not affect the synthesis of mitochondrial proteins versus non-protein control conditions. However, in neither of these previous studies (2, 9) was a resting rate of MitoPS reported and so it is not possible to ascertain whether exercise per se, regardless of the nutrition provided, resulted in a stimulation of MitoPS. Sustained hyperinsulinemia and hyperaminoacidemia support increased MitoPS at rest (32) an effect that appears to require amino acids (1). Whether there is a necessity for protein provision to robustly stimulate MitoPS, as there is with MyoPS (29), is currently unknown. Given the relative size of the skeletal muscle mitochondrial protein pool (4-8%) versus the myofibrillar protein pool (60-70%), it would seem that amino acids would not likely be rate limiting for MitoPS. The physiological relevance of the difference in MitoPS between HIGH and LOW exercise trials in late recovery (i.e. 24 h) is unclear from our acute measures; however, our results may have implications for phenotypic adaptations following a period of chronic training. Further investigations are required to determine: whether amino acid provision alters the exercise-mediated mitochondrial response, particularly in the late phase of recovery, and whether chronic performance of higher versus lower intensities of aerobic exercise (even if energy matched) result in divergent increases in mitochondrial content.

An important regulator of mitochondrial biogenesis, PGC-1α, is thought to coordinate both nuclear and mitochondrial gene expression to induce mitochondrial biogenesis (14). In contrast to previous studies (23, 24), and in line with the absence of an early post-exercise increase in MitoPS, we found that nuclear PGC-1α content was not increased after exercise (Figure 5). The exercise models used in previous studies (23, 24) were of higher intensity than that used in the present study, which may explain the discrepant findings. While exercise intensity-dependent PGC-1α nuclear localization has not been reported, the response of PGC-1α mRNA post-exercise is intensity-dependent, with higher intensity exercise inducing a 2.5-fold greater increase in mRNA 3 h post-exercise than lower intensity exercise (13). Another
factor that may have contributed to the apparent lack of change in PGC-1α nuclear abundance is the timing of biopsy sampling. Nuclear localization of PGC-1α has been observed immediately and 3 h after high-intensity interval exercise (23, 24), which lead to increases in mitochondrial protein content at 24 h post-exercise (23).

In conclusion, we present data demonstrating that MyoPS is elevated early during post-exercise recovery following both HIGH and LOW bouts of aerobic exercise performed in the fasted state; however, only HIGH exercise extended the duration of the elevated MyoPS response. We did not observe an increase in rates of MitoPS or a change in PGC-1α nuclear localization after exercise performed in the fasted state. The greater rates of MitoPS after HIGH as compared to LOW exercise during late post-exercise recovery may serve to enhance the mitochondrial protein pool following chronic training.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Sciences and Engineering Council (NSERC) of Canada and the Canadian Foundation for Innovation to SMP. DMDD was supported by an Ontario Graduate Scholarship, TACV was supported by an NSERC Post-Graduate Scholarship (Doctoral), and DWDW by a Canadian Institutes of Health Research Canada Graduate Scholarship (Doctoral); all wish to acknowledge that source of funding during the conduct of this work.


Table 1. Characteristics of LOW and HIGH intensity exercise trials.

<table>
<thead>
<tr>
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<th>LOW</th>
<th>HIGH</th>
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<tr>
<td>Workload (Watts)</td>
<td>99 ± 4</td>
<td>198 ± 7</td>
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<tr>
<td>Time (min)</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Average %V_\text{O}_2\text{peak}</td>
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<td>76 ± 3*</td>
</tr>
<tr>
<td>Average %HR_{\text{max}}</td>
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<td>Work (kJ)</td>
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Values are means ± SEM. * Significantly different than LOW intensity, $P < 0.001$
**FIGURE LEGENDS**

**Figure 1.** Schematic of the experimental infusion study design. Asterisks represent blood draws and single arrows represent muscle biopsies.

**Figure 2.** Plasma (A and B) and intracellular (C and D) free [ring-$^{13}$C$_6$]-phenylalanine enrichment during early (A and C) and late (B and D) post-exercise recovery. Data are shown as tracer (t) to tracee (T) ratios.

**Figure 3.** Myofibrillar (A) and mitochondrial (B) fractional synthesis rate (% • h$^{-1}$) at rest, and during early and late recovery from HIGH and LOW exercise. *Significantly different from rest ($P<0.05$); † Significantly different from LOW at the same time point ($P<0.05$).

**Figure 4.** Cell signaling molecule phosphorylation (expressed as phosphorylated protein normalized to $\alpha$-tubulin content) of mTOR$^{\text{Ser}2448}$ (A); p70S6K1$^{\text{Thr}389}$ (B), ERK1/2$^{\text{Thr202/Tyr204}}$ (C); p38$^{\text{Thr180/Tyr182}}$ (D); and nuclear PGC-1$\alpha$ content expressed as PGC-1$\alpha$ normalized to histone 2B content. * Significantly different from LOW at time point ($P<0.05$), † Significantly different than all other time points ($P<0.05$).

**Figure 5.** Representative unaltered Western blot images. Spliced portions of the same gel or sample are demarked by black lines around each image portion.

**Figure 6.** Western blot images of MHCI (shown in green), LDH (shown in blue), and COXIV (shown in red) in the myofibrillar, sarcoplasmic, nuclear, and mitochondrial preparations.
Day 1

Primed-constant infusion of L-[ring-\(^{13}\)C]phenylalanine

<table>
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<tr>
<th>Time h</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
<th>1</th>
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<tr>
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Day 2

Primed-constant infusion of L-[ring-\(^{13}\)C]phenylalanine

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</thead>
<tbody>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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Day 3

Primed-constant infusion of L-[ring-\(^{13}\)C]phenylalanine

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</tr>
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Day 4

Primed-constant infusion of L-[ring-\(^{13}\)C]phenylalanine

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<th>1</th>
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<th>4</th>
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