Running Title: PGC-1α isoform and blood flow restriction

Attenuated PGC-1α isoforms following endurance exercise with blood flow restriction

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ABSTRACT

Introduction: Exercise performed with blood flow restriction simultaneously enhances the acute responses to both myogenic and mitochondrial pathways with roles in training adaptation. Methods: We investigated isoform-specific gene expression of the peroxisome proliferator-activated receptor gamma (PPARG) coactivator 1 and selected target genes and proteins regulating skeletal muscle training adaptation. Methods: 9 healthy, untrained males participated of a randomized counter-balanced, cross-over design in which each subject completed a bout of low-intensity endurance exercise performed with blood flow restriction (15 min cycling at 40% of VO_{2peak}, BFR-EE), endurance exercise (30 min cycling at 70% of VO_{2peak}, EE) or resistance exercise (4 x 10 repetitions of leg press at 70% of 1-repetition maximum, RE), followed by one biopsy (*vastus lateralis*) 3 hr after each boat. All exercise sessions were separate by one week. As control a single resting muscle biopsy was obtained two weeks before the first exercise trial (rest). Results: Total PGC-1α mRNA abundance, along with all four isoforms, increased above rest with EE only ($P<0.05$) being higher than BFR-EE ($P<0.05$). PGC-1α1, 2 and 4 were higher after EE compared to RE ($P<0.05$). EE also increased VEGF, Hif-1α and MuRF-1 mRNA abundance above rest ($P<0.05$) while COXIV mRNA expression increased with EE compared to BFR-EE ($P<0.05$). Conclusion: The attenuated expression of all four PGC-1α isoforms when endurance exercise is performed with blood flow restriction suggests this type of exercise provides an insufficient stimulus to activate the signaling pathways governing mitochondrial and angiogenesis responses observed with moderate- to high intensity endurance exercise.

Key words: mitochondrial biogenesis; cell signalling; skeletal muscle; adaptation; angiogenesis; high intensity exercise

Introduction

Skeletal muscle is a highly malleable tissue that can alter its phenotype according to the contractile stimulus imposed (39). For instance, moderate-intensity (i.e., <65% of peak oxygen uptake [VO_{2peak}]) endurance exercise training enhances whole-body $\dot{V}O_{2peak}$ (3, 12), increases the maximal activities of oxidative enzymes, and shifts patterns of substrate selection from carbohydrate- to fat-based fuels (18). In contrast, strenuous (80% of one repetition maximum [1-RM]) resistance exercise has little or no effects on whole-body VO_{2peak} and oxidative enzyme profiles (11) but increases myofibrillar protein accretion and muscle cross-sectional area (CSA) (27).

While resistance and endurance exercise could be considered at opposite ends of the 'adaptation continuum' by virtue of their divergent biochemical and morphological phenotypes, blood flow restriction during low-intensity endurance exercise (BFR-EE) improves both VO_{2peak}, muscle strength and CSA (1, 2). Abe and co-workers (1) reported significant increased, although quite small relative to traditional endurance and resistance exercise, isometric muscle strength, muscle CSA and VO_{2peak} following 8 weeks (24 training sessions) of low-intensity cycle exercise (15 min at 40% VO_{2peak}) performed with BFR-EE compared to same exercise undertaken without BFR. These findings suggest the local hypoxia induced by BFR induces an additive 'metabolic stressor' that perturbs cellular homeostasis (17) and concomitantly enhances both anabolic and oxidative adaptations.

The cellular mechanisms mediating adaptation responses to exercise are complex involving the cross talk of several intracellular signaling systems that ultimately form the basis for specific phenotypic responses with divergent contractile modes (17). The transcriptional co-activator Peroxisome proliferator-activated receptor gamma (PPARG) coactivator 1 alpha (PGC-1α) is a 'master regulator' of many endurance exercise-induced adaptations by virtue of its central role in promoting mitochondrial biogenesis, angiogenesis, and inflammatory proteins (20). Transcription of the PGC-1α gene has been shown to be under the control of several promoter regions with activation of the alternative PGC-1α1 promoter resulting in the transcription of three additional isoforms: PGC-1α2, -α3 and-α4. Ruas and colleagues (30) recently demonstrated a preferential increase in the PGC1-α4 isoform following resistance exercise in human skeletal muscle. However, little is known about the regulation of the α2 and α3 isoforms and, to date, no studies have investigated the expression of all four PGC-1α isoforms to diverse contractile stimuli such as resistance and endurance exercise in humans. Accordingly, the aim of the present study was to compare the acute molecular responses mediated by the different PGC-1α isoforms following low intensity endurance exercise (BFR-EE), resistance exercise (RE) and moderate endurance exercise (EE). As BFR-EE can promote both endurance capacity and muscle hypertrophy responses, we hypothesised EE and RE would selectively increase the expression of the PGC-1α1 and α4 isoforms, respectively. In contrast, we hypothesized that BFR-EE would upregulate a molecular signature involving the increase of both isoforms and their respective anabolic and mitochondrial gene targets.

METHODS

Subjects

Nine untrained, healthy male subjects [age 22.4 ± 3.0 yr, body mass (BM) 73.5 ± 9.7 kg, height 1.79 ± 0.05 m, maximal oxygen uptake test (VO_{2peak}) 36.8 ± 4.8 mLlkg^{-1}·min^{-1}, leg press one repetition maximum (1-RM) 266 ± 66 kg; values are mean ± SD] voluntarily participated in this study. The experimental procedures and possible risks associated with the study were explained to all subjects, who provided written informed consent before participation. The study was approved by the local University's Ethics Committee and conducted in conformity with the policy statement regarding the use of human subjects according to the latest revision of the *Declaration of Helsinki*.

Experimental Design

The study employed a randomized counter-balanced, cross-over design in which each subject completed a bout of either resistance exercise (RE), endurance cycling exercise (EE) or low-intensity cycling exercise combined with blood flow restriction (BFR-EE). Two weeks prior to the first exercise session, a resting muscle biopsy was obtained before participants underwent VO_{2peak} and one-repetition maximum (1-RM) testing, and exercise familiarization. Exercise trials were separated by a one-week recovery period during which time subjects maintained their habitual diet and physical activity patterns.

Preliminary Testing

VO_{2peak}. Participants performed a maximum graded exercise test on a cycle ergometer with electromagnetic braking (Quinton modelo: Corival 400, Lode BV, Groningen, Netherlands). After resting on the bike for 5 min, participants commenced the incremental test protocol. Briefly, subjects commenced cycling at an initial load of 50 W for 1 min and the workload was increased by 15 W/min until a workload of 200

W was reached, after which further increases were 10 W/min increments. The test continued until voluntary exhaustion, defined by two of the three following criteria: VO_{2peak} plateau (< 2.1 $mL.kg^{-1}.min^{-1}$ of variation), > 1.10 respiratory exchange ratio, and/or heart rate higher than 90% of maximum estimated from age (19). Gas exchange data were collected continuously using an automated breath-by-breath metabolic system (CPX, Medical Graphics, St. Paul, Minnesota, USA) and the highest oxygen consumption value was defined as the peak oxygen consumption (VO_{2peak}) over any 30 sec period.

Maximal Strength

The one-repetition maximum (1-RM) test was performed on a leg press machine (45º leg press, G3-PL70; Matrix, São Paulo, Brazil) as previously described (8). Briefly, participants performed a 5 min warm-up on a cycle ergometer riding at 25 W. Participants then undertook 1 x 10 repetitions at 50% of their estimated 1-RM, followed by 1 x 3 repetitions at 70% of the estimated 1-RM with 1-min rest between sets. Participants then performed a series of single repetitions until the maximum load (1-RM) lifted was established with fully eccentric-concentric movement with 90º range of motion. Repetitions were separated by a 3-min recovery and were used to establish the maximum load/weight that could be moved through the full range of motion once, but not a second time.

Diet/Exercise Control

Before each experimental trial (described subsequently), subjects were instructed to refrain from exercise training and vigorous physical activity, and alcohol and caffeine consumption for a minimum of 48 h. Subjects were provided with standardized

prepacked meals that consisted of 3 g carbohydrate/kg body mass (BM), 0.5 g protein/kg BM, and 0.3 g fat/kg body mass consumed as the final caloric intake the evening before reporting for an experimental trial.

Experimental Testing Sessions

On the morning of an experimental trial, subjects reported to the laboratory after a ~10-h overnight fast. After resting in the supine position for ~15 min and under local anaesthesia (2–3 mL of 1% Xylocaine), a resting biopsy was obtained from the *vastus lateralis* using a 5-mm Bergstrom needle modified with suction (7). Approximately 100 mg of muscle was removed, dissected free from blood and connective tissue and snap frozen in liquid nitrogen before being stored at – 80°C until subsequent analyses. Due to ethical constraints regarding the total number of muscle biopsies allowed, this single resting biopsy was used as a basal control for all subsequent exercise trials. Two weeks later participants returned to the laboratory having (after the same pre-trial diet and exercise control) to undertake the first of three randomly assigned exercise sessions (described below). Each exercise trial was separated by a one week wash out. Following the completion of each exercise session, subjects rested for 180 min after which time a muscle biopsy was obtained. Subsequent incisions were performed 3 cm proximal to each other. Blood samples were collected before each exercise session and immediately, 1, 2 hr and 3 hr post exercise. Blood samples were immediately placed in microtubes containing 1% sodium fluoride and then centrifuged at 3000 rpm for 5 min to separate the plasma before being aliquoted and frozen in liquid nitrogen and stored at -80°C.

Resistance Exercise (RE)

After a standardized warm-up on a cycle ergometer consisting of 5 min light cycling at 25 W, subjects performed 4 sets of 10 repetitions leg press exercise (45° leg press machine; G3-PL70; Matrix) at 70% of 1-RM. Each set was separated by a 1 min recovery period during which time subjects remained seated on the leg press machine. Complete concentric/eccentric movements were performed with 90° of range of motion and strong verbal encouragement was provided during each set.

Endurance Exercise (EE)

Following a standardized warm up (described previously), subjects performed 30 min of continuous cycling at a power output that elicited ~at 70% of individual VO_{2peak}. Subjects were fan-cooled and provided visual feedback for pedal frequency, power output, and elapsed time were provided to subjects.

*Low Intensity Blood Flow Restriction (*BFR-EE*)*

Subjects performed 15 min continuous cycling with a cuff strapped over the thigh at a power output that elicited at 40% of VO_{2peak}, as previously reported (1). An 18-cm wide cuff was placed on the proximal portion of the thigh (inguinal fold region) over the tibial artery (38) and once in position, was inflated until an absence of auditory blood pulse detected through auscultation with a vascular Doppler probe (DV-600; Marted, São Paulo, Brazil). Pressure was then slowly released until the first arterial pulse was detected which was considered the systolic pressure at the tibial artery. Cuff pressure was set at 80% of the maximum tibial arterial pressure and the cuff was inflated through-out the entire exercise session.

Analytical Procedures

Blood Lactate

Plasma lactate concentration was measured on a spectrophotometer (ELx800, Biotek, Winooski, USA) using a commercial kit (Biotecnica, Varginha, Brazil) according to the manufacturer's protocol.

RNA Extraction and Quantification

Approximately 20 mg of skeletal muscle was homogenized in TRIzol with chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with ice-cold isopropanol alcohol and the resulting pellet was washed and re-suspended in 40 µl of RNase-free water. Extracted RNA was quantified using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) by measuring absorbance at 260 nm and 280 nm.

Reverse Transcription

First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial dilutions of a template human skeletal muscle RNA (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR).

Real-Time PCR

Quantification (in duplicate) of mRNA was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, California, USA). Taqman-FAM-labelled primer/probes for MuRF-1 (Cat No. Hs00822397_m1), COXIV (Cat No. Hs00971639_m1), IL-6 (Cat No. Hs00985639_m1), PGC-1α (Cat No. Hs01016719_m1), HIF-1α (Cat No. Hs00153153_m1), Myostatin (Hs00976237_m1), IGF-1 (Hs01547656_m1) and VEGF (Cat No. Hs00900055_m1) were used in a final reaction volume of 20 µL. PCR treatments were 2 min at 50 ºC for UNG activation, 10 min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat No Hs02758991_g1) was used as a housekeeping gene and was stably expressed between exercise interventions (data not shown). The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (22).

Quantification of PGC-1α isoforms

RNA was extracted from a separate piece of snap frozen muscle (~20 mg) using TRIzol (Invitrogen) and purified using QIAGEN RNeasy mini-columns. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Bio-systems). Real-Time Quantitative PCR was carried out in a SYBR Green ER PCR Master Mix (Invitrogen)/ 384-well format using an ABI PRISM 7900HT (Applied Biosystems). Relative mRNA levels were calculated using the comparative CT method and normalized to cyclophilin mRNA. Primer sequences are as follows: Cyclophilin (forward: GGAGATGGCACAGGAGGAA; reverse: GCCCGTAGTGC TTCAGTTT), PGC1α1 (forward: ATG GAG TGA CAT CGA GTG TGC T; reverse: GAG TCC ACC CAG AAA GCT GT), PGC1α2 (forward: AGT CCA CCC AGA AAG CTG TCT; reverse: ATG AAT GAC ACA CAT GTT GGG), PGC1α3 (forward: CTG CAC CTA

GGA GGC TTT ATG C; reverse: CAA TCC ACC CAG AAA GCT GTC T), and PGC1α4 (forward: TCA CAC CAA ACC CAC AGA GA; reverse: CTG GAA GAT ATG GCA CAT).

Western Blots

Approximately 30 mg of muscle was homogenized in buffer containing 50 mM Tris·HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10% μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1mM benzamidine, and 1 mM PMSF. After determination of protein concentration (Pierce, Rockford, IL), lysate was resuspended in Laemmli sample buffer. Lysate was then re-suspended in Laemmli sample buffer with 40 μg of protein loaded onto 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (Bio Rad, California, USA). Post electrophoresis gels were activated according to the manufacturer's details (Chemidoc, Bio-Rad, Gladesville, Australia) and then transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a Stain-Free image of the PVDF membranes for total protein normalization was obtained before membranes were rinsed briefly in distilled water and blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry. All sample time points for each subject were run on the same gel. Polyclonal anti-phospho-mTOR^{Ser2448} (no. 2971), -p70 S6K^{Thr389} (no. 9206), - adenosine monophosphate kinase (AMPK)^{Thr172} (no. 2531), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) 4E-BP1$^{Thr37/46}$ (no. 9459), eEF2 eukaryotic translation elongation factor 2 (eEF2) eEF2^{Thr56}

(no. 2331) and $p53^{Ser15}$ (no. 9284) were purchased from Cell Signaling Technology (Danvers, MA, USA). Volume density of each target protein band was normalized to the total protein loaded into each lane using stain-free technology (15) with data expressed in arbitrary units.

Statistical analysis

Statistical analysis was performed using SAS version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA). Data normality and variance equality were assessed through the Shapiro-Wilk and Levene tests. One-way ANOVA with repeated measures (factor: condition) was performed for gene and protein expression analyses. A mixed model ANOVA, assuming group and time as fixed factors and subjects as a random factor, was performed for blood lactate data. Tukey post hoc analysis was used for multiple comparison purposes when significant F-values were found. The significance level was set at $P \leq 0.05$. Data are presented as Mean ± Standard Deviation (SD).

Results

mRNA expression

Total PGC1-α and isoforms

Total PGC-1α mRNA (Figure 1A) increased with EE above rest (P<0.0001), RE (P=0.0013) and BFR-EE (P>0.0001). There was a significant increase in PGC-1α1 mRNA with EE above rest (P=0.0450), RE (P=0.0069) and BFR-EE (P=0.0349) (Figure 1B). There was also a significant increase in PGC-1α2 mRNA (Figure 1C) with EE above rest (P<0.0001), RE (P=0.0003) and BFR-EE (P<0.0001). PGC-1α3 mRNA (Figure 1D) increased with EE above rest (P=0.0389). There was also increases PGC-1α4 mRNA (Figure 1E) with EE above rest (P=0.0035), RE (P=0.0469) and BFR-EE

(P=0.0140).

Figure 1 here

VEGF, COXIV, HIF-1a

There was a significant increase in VEGF mRNA (Figure 2A) with EE above rest (P=0.0180) and RE (P=0.0069). COXIV mRNA expression increased with EE above BFR-EE (P=0.0550) (Figure 2B). There was a significant increase in HIF-1a abundance with EE above Rest (P= 0.0530) (Figure 2C).

Figure 2 here

IL-6 – IGF-1 - Myostatin - MurRF1

IL-6, IGF-1 and Myostatin mRNA expression were unchanged post-exercise (Figure 3 A, B, C). There was a post-exercise increase in MuRF1 mRNA abundance with EE above Rest (P=0.0003), RE (P=0.0256) and BFR-EE (P=0.0007) (Figure 3D).

Figure 3 here

Cell Signaling

mTOR -p70S6K -4E-BP1 -eEF2

There were no changes in mTOR$^{\mathbf{Ser2448}}$, p70S6K^{Thr389}, 4E-BP1$^{Thr37/46}$or eEF2^{Thr56} phosphorylation post-exercise or between exercise groups (Figure 4).

Figure 4 here

AMPK -p53

AMPK^{Thr156}and p53^{Ser15} phosphorylation were unchanged post-exercise (Figure 5).

Figure 5 here

Plasma lactate concentration

Lactate concentration increased above rest immediately post-exercise for all interventions (P<.0001 for all comparisons; Table 1). Lactate concentration remained elevated at 1 h, 2 h and 3 h post-exercise for EE and RE, and 1 h and 2 h for BFR-EE (P<.0001 for all comparisons).

Table 1 here

Discussion

It has been shown that, although quite small relative to traditional endurance and resistance exercise, Low intensity (<50% of VO_{2peak}) endurance training with blood flow restriction concomitantly promote isometric muscle strength, muscle CSA and VO_{2peak} (1, 2) and , while the underlying molecular mechanisms mediating these responses remain largely undefined. For the first time we report that low intensity endurance cycling exercise performed with blood flow restriction failed to increase PGC-1α expression to that commonly observed with 'conventional' endurance exercise.

Moreover, we show isoform-specific post-exercise increases in the α4 isoform along with Hif-1α and VEGF mRNA expression following higher intensity endurance exercise without blood flow restriction. Taken collectively, our novel findings suggest that endurance exercise undertaken with blood flow restriction is unable to provoke the perturbations to cellular homeostasis necessary to induce activation of the cell signaling events regulating mitochondrial biogenesis and angiogenesis that take place with higher intensity endurance exercise without blood flow restriction.

A growing body of evidence suggests that exercise undertaken with blood flow restriction can enhance exercise adaptation. A recent meta-analysis reported both low load/intensity resistance (20–30% 1 RM) and aerobic walking exercise performed with blood flow restriction can induce increases in muscle strength and hypertrophy, although with smaller gains compared to high intensity resistance exercise alone (34). However, little is known about the molecular mechanisms mediating these responses when low intensity endurance exercise is undertaken with blood flow restriction. As such, we compared the expression of key gene and protein targets implicated in a range of exercise adaptation responses such as hypertrophy, mitochondrial biogenesis, muscle proteolysis, substrate metabolism and angiogenesis between BFR-EE, and conventional bouts of RE and EE. We particularly focused on the four different full-length PGC-1α isoforms putatively implicated in anabolic and mitochondrial-related adaptation responses.

In agreement with previous studies (5, 21, 26), we observed significant increases in total PGC-1α mRNA following continuous endurance exercise performed at 70% of VO_{2peak}. This increase in PGC-1α mRNA was concomitant with greater abundance of VEGF, a

target of PGC-1α (36). However, in contrast to our original hypothesis, this response was absent following a bout of low-intensity endurance exercise (40% VO_{2peak}) performed with blood flow restriction. In an attempt to identify possible mechanisms responsible for this attenuated PGC-1α response, we investigated IL-6 expression to determine whether an increase in the muscular inflammatory program was implicated in the blunted response. This hypothesis was based on previous data showing an inverse relationship between skeletal muscle PGC-1α and IL-6 expression (16). However, IL-6 mRNA expression post-exercise was unchanged in all exercise groups suggesting any acute increase in muscle inflammation caused by BFR-EE was not responsible for the reduced PGC-1α1 expression observed. We also investigated other cellular markers implicated in exercise adaptation responses that can regulate PGC-1α expression. AMPK is an intracellular 'fuel gauge' that can phosphorylate PGC-1α and increase its transcriptional activity (35) while the apoptogenic protein p53 has emerged as another signaling regulator of skeletal muscle exercise-induced mitochondrial biogenesis and substrate metabolism that can translocate to the nucleus upon activation and induce PGC-1α expression (4). Phosphorylation of either of these protein targets was unaltered post-exercise suggesting other molecular markers and/or physiological mechanisms may be responsible for the upregulation of PGC-1α with high intensity endurance exercise. One plausible explanation for these discrepant findings may be the level of glycogen utilization between exercise sessions in our untrained subjects. We (10) and others (6, 29) have shown greater post-exercise PGC-1α expression with low- compared to normal or high glycogen concentration and although we did not measure muscle glycogen use in the current study due to limited muscle tissue availability, the longer duration and higher intensity exercise bout is likely to have induced greater glycogen

395 depletion compared to the endurance exercise session performed with blood flow
396 restriction.
397
398 Another possible explanation for the discrepancy in PGC-1α1 expression between the
399 two endurance-based exercise bouts is the large differences in estimated energy
400 expenditure. Exercise energy expenditure after BFR-EE was ~4 fold less compared to
401 the EE protocol with total energy expenditure positively associated with PGC-1α
402 expression ($r=0.73$, $P=0.039$). Increased PGC-1α mRNA expression has been observed
403 after 30 min running compared to bouts of 20 and 10 min (36). Thus, total exercise-
404 induced energy expenditure may be an overriding determinant of PGC-1α expression
405 responses post-exercise.
406
407 Low intensity endurance exercise with BFR was also unable to induce the expression of
408 PGC-1α4 compared to higher intensity endurance exercise without blood flow
409 restriction. The PGC-1α4 isoform has been proposed to promote muscle hypertrophy by
410 inducing IGF-1 expression and reducing the expression of myostatin, a negative
411 regulator of muscle growth (30). The increase in PGC-1α4 mRNA expression with EE
412 was mirrored by a small, non-significant, increase and decrease in IGF1 and myostatin
413 expression, respectively. Other studies have also observed increases in PGC-1α4
414 expression with endurance exercise (33, 40) suggesting this isoform can be regulated
415 with acute exercise independent of contractile mode.
416
417 Increased PGC-1α4 and VEGF expression has also been reported in primary myotubes
418 treated under hypoxic conditions suggesting low oxygen conditions to be favorable for
419 the activation of this isoform (37). In the current study, the transcription factor Hif-1α, a

key regulator of angiogenesis in situations of hypoxia (32), was unchanged following BFR-EE, while RE and EE induced 2-fold higher post-exercise changes in lactate compared to BFR-EE. While it is possible a greater metabolic and hypoxic stimulus may be required to increase PGC-1α4 signaling, others have reported unchanged blood lactate following aerobic-based exercise with blood flow restriction (23). Moreover, the same occlusion protocol (15 min cycle at 40% VO_{2peak}) has been shown to improve muscle volume and VO_{2peak}, during a chronic training intervention (1). Thus, it is possible chronic exposure to this occlusion stimulus may be required to elicit increases in PGC-1α4 expression. As this is the first study to investigate changes in Hif-1α following endurance cycling exercise with BFR it is difficult to compare our results to those of previous investigations incorporating resistance exercise and BFR. However, we speculate that when performed with blood flow restriction, the lower contractile intensity associated with 'conventional' endurance compared to resistance (or sprint) exercise, provides adequate blood flow to the exercising musculature and adjoining capillary beds in order to prevent tissue de-oxygenation. Further studies comparing different low intensity endurance exercise protocols with resistance exercise that incorporate blood flow restriction are required to corroborate this hypothesis.

Another novel finding from the current study was the post-exercise increases in the PGC-1α2 and 3 isoforms. Similar to the α1 and α4 isoforms, both PGC-1α2 and α3 increased above rest with higher intensity endurance exercise and were significantly elevated compared to resistance exercise. Both isoforms are expressed in skeletal muscle and brown adipose tissue although little is known about the regulatory targets of these isoforms and their capacity to mediate exercise adaptation responses. Accordingly with Ruas et al (2012) the functions of PGC-1α2 and α3 remain under investigation,

445 however, based on the elevated response following endurance compared to resistance
446 exercise, we propose these isoforms to mediate physiological processes related to
447 mitochondrial biogenesis and substrate metabolism.
448
449 Considering low load endurance exercise with BFR can increase muscle strength and
450 hypertrophy (34), we also investigated markers of translation initiation, elongation and
451 muscle proteolysis. Previous studies have reported increases in mTOR and p70S6K
452 phosphorylation that have formed the basis for enhanced rates of muscle protein
453 synthesis following resistance exercise with blood flow restriction (13, 14).
454 Nonetheless, the phosphorylation status of these proteins as well as 4E-BP1 and eEF2
455 were unchanged 3 h post-exercise in the current study. While our study design was
456 somewhat limited by only having the single post-exercise biopsy (9), this sampling
457 time-point was specifically chosen based on previous studies showing significant, and
458 in some cases maximal, increases in PGC-1α mRNA expression in response to an
459 exercise challenge (5, 21, 24, 28). Accordingly, Ozaki et al.,(25) investigated molecular
460 effects of walking exercise with BFR on mTOR and downstream targets and although
461 the walking exercise with BFR was performed with higher intensity (55% of VO2peak)
462 compared to our protocol, it was not found any significant modification to mTOR
463 signaling. As in our study, probably the biopsy time point was the cause of absence of
464 significant mTOR and downstream targets difference. Thus, future studies investigating
465 endurance exercise undertaken with BFR-EE should include a time-course of signaling
466 responses in order to determine the optimal 'window' for muscle sampling in
467 subsequent investigations. MuRF-1 mRNA expression increased post endurance
468 exercise which resulted in a higher expression above endurance exercise with BFR and
469 resistance exercise. MuRF-1 mediates the ubiquitin proteasome system by 'labelling'

cleaved myofibril segments for degradation (31). It is unclear whether this increase in expression with high intensity endurance exercise represents general tissue remodeling, particularly considering our participants were untrained and the unaccustomed contractile stimulus, or a greater induction of protein degradation.

In summary, this is the first study to investigate the molecular mechanisms mediating muscle adaptation responses to low intensity endurance cycling exercise with blood flow restriction. The attenuated expression of all four PGC-1α isoforms when endurance exercise is performed with blood flow restriction suggests this type of exercise is unable to induce the appropriate metabolic perturbation capable of activating the cell signaling machinery responsible for mitochondrial biogenesis and angiogenesis responses with moderate-to-high intensity endurance exercise. Longer training programs incorporating endurance exercise with BFR that correlate measurements of these molecular markers with functional adaptation responses such as changes in VO_{2peak} and cycle time to fatigue will yield important information to the efficacy of this training method to enhance training adaptation and subsequently improve health outcomes in populations that may be unable to perform, prolonged exercise.

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Figure 1. (A) Total Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), (B) Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α1), (C) Peroxisome proliferator-activated receptor-γ coactivator 1α2 (PGC-1α2), (D) Peroxisome proliferator-activated receptor-γ coactivator 1α3 (PGC-1α3) and (E) Peroxisome proliferator-activated receptor-γ coactivator 1α4 (PGC-1α4) mRNA abundance at rest and 3 h post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or low-intensity associated with blood flow restriction (BFR-EE). Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n=9). a= Significant different from Rest ($P \leq 0.05$); b= Significant different from HI-RT ($P \leq 0.05$); c= Significant different from BFR-EE ($P \leq 0.05$).

Figure 2. (A) Vascular endothelial growth factor (VEGF), (B) Cytochrome c oxidase subunit 4 isoform 1 (COXIV) and (C) hypoxia-inducible factor-1 alpha (HIF-1α) mRNA abundance at rest and 3 h post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or low-intensity associated with blood flow restriction (BFR-EE). Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n=9). a= Significant different from Rest ($P \leq 0.05$); b= Significant different from HI-RT ($P \leq 0.05$); c= Significant different from BFR-EE ($P \leq 0.05$).

Figure 3. (A) Interleukin 6 (IL-6), (B) Insulin-like growth factor 1(IGF-1), (C) Muscle RING finger 1 (MURF1) and (D) Myostatin mRNA abundance at rest and 3 h post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or low-intensity associated with blood flow restriction (BFR-EE). Values are expressed relative to GAPDH and presented in arbitrary units (mean ± SD, n=9). a= Significant different from Rest ($P \leq 0.05$); b= Significant different from HI-RT ($P \leq 0.05$); c= Significant different from BFR-EE ($P \leq 0.05$).

Figure 4. (A) Mechanistic target of rapamycin (mTOR)^{Ser2448} (B) p70S6K^{Thr389} (C) eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)$^{Thr37/46}$ eukaryotic elongation factor 2 (eEF2)^{Thr56} phosphorylation in skeletal muscle at rest and after 3 h post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or low-intensity associated with blood flow restriction (BFR-EE). Values are normalized to total protein loaded determined by stain free technology in arbitrary units (mean ± SD, n=9).

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719 **Figure 5.** (A) Adenosine Monophosphate-Activated Protein (AMPK)^{Thr172} and (B)
720 p53^{Ser15} phosphorylation in skeletal muscle at rest and after 3 h post-exercise recovery
721 following endurance exercise (EE), resistance exercise (RE) or low-intensity associated
722 with blood flow restriction (BFR-EE). Values are normalized to total protein loaded
723 determined by stain free technology in arbitrary units (mean ± SD, n=9).

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