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. 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 in 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) separated by at least one-week recovery. A single resting muscle biopsy (*vastus lateralis*) was obtained two weeks before the first exercise trial (rest) and 3 h after each boat. 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 $\dot{V}O_{2peak}$ (4), 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) (30).

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 increased 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. While these adaptation responses are considerably lower in magnitude relative to conventional endurance and resistance training performed at higher intensities, the local hypoxia induced by BFR appears to induce 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 (17). 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 (32) 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, or following BFR, 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 $mLlkg^{-1}\cdot min^{-1}$, 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) based on a protocol used in previously published paper that investigated BFR-EE (1). Briefly, 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:

126 VO_{2peak} plateau (< 2.1 $mL.kg^{-1}.min^{-1}$ of variation), > 1.10 respiratory exchange ratio,
127 and/or heart rate higher than 90% of maximum estimated from age (19). Gas exchange
128 data were collected continuously using an automated breath-by-breath metabolic system
129 (CPX, Medical Graphics, St. Paul, Minnesota, USA) and the highest oxygen
130 consumption value was defined as the peak oxygen consumption (VO_{2peak}) over any 30
131 sec period. To confirm the appropriateness of this protocol for this study we performed
132 a pilot study to verify repeatability in VO_{2peak} measures and observed a strong
133 repeatability in VO_{2peak} (3.0%), power (1.9%), respiratory exchange ratio (RER) (5.6%),
134 and time to exhaustion (1.6%) measures.
135
136 *Maximal Strength*
137 The one-repetition maximum (1-RM) test was performed on a leg press machine
138 (45º leg press, G3-PL70; Matrix, São Paulo, Brazil) as previously described (8). Briefly,
139 participants performed a 5 min warm-up on a cycle ergometer riding at 25 W.
140 Participants then undertook 1 x 10 repetitions at 50% of their estimated 1-RM, followed
141 by 1 x 3 repetitions at 70% of the estimated 1-RM with 1-min rest between sets.
142 Participants then performed a series of single repetitions until the maximum load (1-
143 RM) lifted was established with fully eccentric-concentric movement with 90º range of
144 motion. Repetitions were separated by a 3-min recovery and were used to establish the
145 maximum load/weight that could be moved through the full range of motion once, but
146 not a second time.
147
148 *Diet/Exercise Control*
149 Before each experimental trial (described subsequently), subjects were instructed
150 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. The volume and intensity of this session was based on the recommendations of American College Sports Medicine (ACSM) (3). All participants completed every repetition from each respective 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. The volume and intensity of this session were based on the recommendations of ACSM (4). All participants completed the full 30 min session.

*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 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 (22).

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 (24). All Taqman-based PCR experiments were performed in the Centre for Exercise and Nutrition laboratory at the Australian Catholic University.

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). All SYBR Green-based PCR experiments were performed in the Department of Cell Biology laboratory at the Dana-Farber Cancer Institute, Harvard Medical School (USA).

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. Due to low sample availability, phosphorylated proteins were unable to be normalized to their respective total protein content and were therefore also normalized to stain-free protein levels.

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

Low intensity (<50% of VO_{2peak}) endurance training with blood flow restriction has been shown to concomitantly promote isometric muscle strength, muscle CSA and VO_{2peak} (1, 2). While these enhanced adaptation responses are considerably lower in magnitude compared to conventional resistance or endurance exercise performed without any blood flow restriction, 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 cycle 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 (35). However, little is known about the molecular mechanisms mediating these responses

374 when low intensity endurance exercise is undertaken with blood flow restriction. As
375 such, we compared the expression of key gene and protein targets implicated in a range
376 of exercise adaptation responses such as hypertrophy, mitochondrial biogenesis, muscle
377 proteolysis, substrate metabolism and angiogenesis between BFR-EE, and conventional
378 bouts of RE and EE. We particularly focused on the four different full-length PGC-1α
379 isoforms putatively implicated in anabolic and mitochondrial-related adaptation
380 responses.
381
382 In agreement with previous studies (5, 23, 29), we observed significant increases in total
383 PGC-1α mRNA following continuous endurance exercise performed at 70% of VO_{2peak}.
384 This increase in PGC-1α mRNA was concomitant with greater abundance of VEGF, a
385 target of PGC-1α (37). However, in contrast to our original hypothesis, this response
386 was absent following a bout of low-intensity endurance exercise (40% VO_{2peak})
387 performed with blood flow restriction. In an attempt to identify possible mechanisms
388 responsible for this attenuated PGC-1α response, we investigated IL-6 expression to
389 determine whether an increase in the muscular inflammatory program was implicated in
390 the blunted response. This hypothesis was based on previous data showing an inverse
391 relationship between skeletal muscle PGC-1α and IL-6 expression (16). However, IL-6
392 mRNA expression post-exercise was unchanged in all exercise groups suggesting any
393 acute increase in muscle inflammation caused by BFR-EE was not responsible for the
394 reduced PGC-1α1 expression observed. We also investigated other cellular markers
395 implicated in exercise adaptation responses that can regulate PGC-1α expression.
396 AMPK is an intracellular ‘fuel gauge’ that can phosphorylate PGC-1α and increase its
397 transcriptional activity (36) while the apoptogenic protein p53 has emerged as another
398 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 (17). 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, 31) 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 depletion compared to the endurance exercise session performed with blood flow restriction.

Another possible explanation for the discrepancy in PGC-1α1 expression between the two endurance-based exercise bouts is the large differences in estimated energy expenditure. Exercise energy expenditure after BFR-EE was ~4 fold less compared to the EE protocol with total energy expenditure positively associated with PGC-1α expression ($r=0.73$, $P=0.039$). Increased PGC-1α mRNA expression has been observed after 30 min running compared to bouts of 20 and 10 min (37). Thus, total exercise-induced energy expenditure may be an overriding determinant of PGC-1α expression responses post-exercise.

Low intensity endurance exercise with BFR was also unable to induce the expression of PGC-1α4 compared to higher intensity endurance exercise without blood flow restriction. The PGC-1α4 isoform has been proposed to promote muscle hypertrophy by

inducing IGF-1 expression and reducing the expression of myostatin, a negative regulator of muscle growth (32). The increase in PGC-1α4 mRNA expression with EE in the current study was mirrored by a small, non-significant, increase and decrease in IGF1 and myostatin expression, respectively. Ruas and colleagues were the first to show a selective increase in PGC-1α4 expression (concomitant with decreased myostatin abundance) with resistance compared to endurance exercise in human skeletal muscle (32). However, this expression pattern was observed following 8 weeks whole-body resistance training. Thus, a limitation of our study is that we only incorporated a single bout of isolated leg press suggesting longer training programs/ exercise stimulus may be required to induce this selective PGC-1α4 response. Nonetheless, another recent publication reported increased truncated and non-truncated PGC-1a transcripts from both alternative and proximal promoter sites 2 hours following an acute bout of resistance exercise that incorporated the same volume and intensity as our study (40). This indicates the resistance exercise bout performed in our study was likely sufficient to induce the appropriate signal to increase the expression of this isoform however potential differences in post-exercise biopsy timing between this study and ours (2 h vs. 3 h) may explain why we did not observe this increase with resistance exercise.

Increased PGC-1α4 and VEGF expression has also been reported in primary myotubes treated under hypoxic conditions suggesting low oxygen conditions to be favorable for the activation of this isoform (38). In the current study, the transcription factor Hif-1α, a key regulator of angiogenesis in situations of hypoxia (34), 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 (26). 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 (27). Based on the elevated response following endurance compared to resistance exercise, we propose these isoforms to mediate physiological processes related to mitochondrial biogenesis and substrate metabolism.

Considering low load endurance exercise with BFR can increase muscle strength and hypertrophy (35), we also investigated markers of translation initiation, elongation and muscle proteolysis. Previous studies have reported increases in mTOR and p70S6K phosphorylation that have formed the basis for enhanced rates of muscle protein synthesis following resistance exercise with blood flow restriction (13, 14). Nonetheless, the phosphorylation status of these proteins as well as 4E-BP1 and eEF2 were unchanged 3 h post-exercise in the current study. This is in agreement with the results of Ozaki and colleagues (28) who observed no changes in Akt, mTOR or p70S6K phosphorylation following 20 min treadmill walking performed with blood flow restriction despite a higher intensity exercise bout (55% VO_{2peak}) compared to our protocol. While our study design was somewhat limited by only having the single post-exercise biopsy (9), this sampling time-point was specifically chosen based on previous studies showing significant, and in some cases maximal, increases in PGC-1α mRNA expression in response to an exercise challenge (5, 23). Future studies investigating endurance exercise undertaken with BFR-EE should include a time-course of signaling responses in order to determine the optimal 'window' for muscle sampling in subsequent investigations.

Several other factors including the width and pressure of cuff used during BFR must also be considered. Previous studies have reported smaller increases in muscle CSA when lower body resistance training is undertaken with BFR (compared to no BFR) at the site of the cuff (12, 20). While this indicates a narrow cuff may be advantageous for promoting anabolic adaptation responses due to compressing less muscle tissue, a recent study comparing the effects of a wide versus narrow cuff reported similar increases in maximum strength and muscle cross sectional area following 12 weeks of unilateral

498 elbow flexion performed at 20% of 1RM (21). Also, a recent study showed that there
499 was no difference in either muscle strength or hypertrophy between different occlusion
500 pressures (25). Thus, the use of a wider cuff, as used in our protocol, appears unlikely to
501 attenuate chronic muscle anabolic responses. Regardless, these studies are currently
502 only limited to BFR with resistance exercise. Future studies comparing these parameters
503 when endurance exercise is performed with BFR are required. Finally, MuRF-1 mRNA
504 expression increased post endurance exercise which resulted in a higher expression
505 above endurance exercise with BFR and resistance exercise. MuRF-1 mediates the
506 ubiquitin proteasome system by 'labelling' cleaved myofibril segments for degradation
507 (33). It is unclear whether this increase in expression with high intensity endurance
508 exercise represents general tissue remodeling, particularly considering our participants
509 were untrained and the unaccustomed contractile stimulus, or a greater induction of
510 protein degradation.
511
512 In summary, this is the first study to investigate the molecular mechanisms mediating
513 muscle adaptation responses to low intensity endurance cycling exercise with blood
514 flow restriction. The attenuated expression of all four PGC-1α isoforms when endurance
515 exercise is performed with blood flow restriction suggests this type of exercise is unable
516 to induce the appropriate metabolic perturbation capable of activating the cell signaling
517 machinery responsible for mitochondrial biogenesis and angiogenesis responses with
518 moderate-to-high intensity endurance exercise. Longer training programs incorporating
519 endurance exercise with BFR that correlate measurements of these molecular markers
520 with functional adaptation responses such as changes in VO_{2peak} and cycle time to
521 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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References

1. Abe T, Fujita S, Nakajima T et al. Effects of Low-Intensity Cycle Training with Restricted Leg Blood Flow on Thigh Muscle Volume and VO2MAX in Young Men. *J Sports Sci Med*. 2010;9(3):452-8.
2. Abe T, Kearns CF, Sato Y. Muscle size and strength are increased following walk training with restricted venous blood flow from the leg muscle, Kaatsu-walk training. *J Appl Physiol (1985)*. 2006;100(5):1460-6.
3. ACSM. American College of Sports Medicine position stand. Progression models in resistance training for healthy adults. *Med Sci Sports Exerc*. 2009;41(3):687-708.
4. ACSM. American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise. *Med Sci Sports Exerc*. 2011;43(7):1334-59.
5. Bartlett JD, Hwa Joo C, Jeong TS et al. Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol (1985)*. 2012;112(7):1135-43.
6. Bartlett JD, Louhelainen J, Iqbal Z et al. Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. *Am J Physiol Regul Integr Comp Physiol*. 2013;304(6):R450-8.
7. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scandinavian journal of clinical and laboratory investigation*. 1975;35(7):609-16.

572 8. Brown LE, Weir JP. Procedures recommendation I: Accurate assessment of
573 muscular strength and power. *Journal of Exercise Physiology Online*. 2001;4:1-
574 21.
575 9. Camera DM, Edge J, Short MJ, Hawley JA, Coffey VG. Early time course of
576 Akt phosphorylation after endurance and resistance exercise. *Med Sci Sports*
577 *Exerc*. 2010;42(10):1843-52.
578 10. Camera DM, Hawley JA, Coffey VG. Resistance exercise with low glycogen
579 increases p53 phosphorylation and PGC-1alpha mRNA in skeletal muscle. *Eur J*
580 *Appl Physiol*. 2015;115(6):1185-94.
581 11. Campos GE, Luecke TJ, Wendeln HK et al. Muscular adaptations in response to
582 three different resistance-training regimens: specificity of repetition maximum
583 training zones. *Eur J Appl Physiol*. 2002;88(1-2):50-60.
584 12. Ellefsen S, Hammarstrom D, Strand TA et al. Blood flow-restricted strength
585 training displays high functional and biological efficacy in women: a within-
586 subject comparison with high-load strength training. *Am J Physiol Regul Integr*
587 *Comp Physiol*. 2015;309(7):R767-79.
588 13. Fry CS, Glynn EL, Drummond MJ et al. Blood flow restriction exercise
589 stimulates mTORC1 signaling and muscle protein synthesis in older men. *J Appl*
590 *Physiol*. 2010;108(5):1199-209.
591 14. Gundermann DM, Walker DK, Reidy PT et al. Activation of mTORC1 signaling
592 and protein synthesis in human muscle following blood flow restriction exercise
593 is inhibited by rapamycin. *Am J Physiol Endocrinol Metab*.
594 2014;306(10):E1198-204.
595 15. Gurtler A, Kunz N, Gomolka M et al. Stain-Free technology as a normalization
596 tool in Western blot analysis. *Anal Biochem*. 2013;433(2):105-11.

16. Handschin C, Choi CS, Chin S et al. Abnormal glucose homeostasis in skeletal muscle-specific PGC-1alpha knockout mice reveals skeletal muscle-pancreatic beta cell crosstalk. *The Journal of clinical investigation*. 2007;117(11):3463-74.

17. Hawley JA, Hargreaves M, Joyner MJ, Zierath JR. Integrative biology of exercise. *Cell*. 2014;159(4):738-49.

18. Holloszy JO, Booth FW. Biochemical adaptations to endurance exercise in muscle. *Annual review of physiology*. 1976;38:273-91.

19. Howley ET, Bassett DR, Jr., Welch HG. Criteria for maximal oxygen uptake: review and commentary. *Med Sci Sports Exerc*. 1995;27(9):1292-301.

20. Kacin A, Strazar K. Frequent low-load ischemic resistance exercise to failure enhances muscle oxygen delivery and endurance capacity. *Scand J Med Sci Sports*. 2011;21(6):e231-41.

21. Laurentino GC, Loenneke JP, Teixeira EL, Nakajima E, Iared W, Tricoli V. The Effect of Cuff Width on Muscle Adaptations after Blood Flow Restriction Training. *Med Sci Sports Exerc*. 2015.

22. Laurentino GC, Ugrinowitsch C, Roschel H et al. Strength training with blood flow restriction diminishes myostatin gene expression. *Med Sci Sports Exerc*. 2012;44(3):406-12.

23. Little JP, Safdar A, Bishop D, Tarnopolsky MA, Gibala MJ. An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol*. 2011;300(6):R1303-10.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-8.

622 25. Lixandrao ME, Ugrinowitsch C, Laurentino G et al. Effects of exercise intensity
623 and occlusion pressure after 12 weeks of resistance training with blood-flow
624 restriction. *Eur J Appl Physiol*. 2015.
625 26. Loenneke JP, Thrower AD, Balapur A, Barnes JT, Pujol TJ. Blood flow-
626 restricted walking does not result in an accumulation of metabolites. *Clin*
627 *Physiol Funct Imaging*. 2012;32(1):80-2.
628 27. Martinez-Redondo V, Pettersson AT, Ruas JL. The hitchhiker's guide to PGC-
629 1alpha isoform structure and biological functions. *Diabetologia*.
630 2015;58(9):1969-77.
631 28. Ozaki H, Kakigi R, Kobayashi H, Loenneke JP, Abe T, Naito H. Effects of
632 walking combined with restricted leg blood flow on mTOR and MAPK
633 signalling in young men. *Acta Physiol (Oxf)*. 2014;211(1):97-106.
634 29. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL.
635 Repeated transient mRNA bursts precede increases in transcriptional and
636 mitochondrial proteins during training in human skeletal muscle. *J Physiol*.
637 2010;588(Pt 23):4795-810.
638 30. Phillips SM. Physiologic and molecular bases of muscle hypertrophy and
639 atrophy: impact of resistance exercise on human skeletal muscle (protein and
640 exercise dose effects). *Appl Physiol Nutr Metab*. 2009;34(3):403-10.
641 31. Psilander N, Frank P, Flockhart M, Sahlin K. Exercise with low glycogen
642 increases PGC-1alpha gene expression in human skeletal muscle. *Eur J Appl*
643 *Physiol*. 2013;113(4):951-63.
644 32. Ruas JL, White JP, Rao RR et al. A PGC-1alpha isoform induced by resistance
645 training regulates skeletal muscle hypertrophy. *Cell*. 2012;151(6):1319-31.

33. Sanchez AM, Candau RB, Bernardi H. FoxO transcription factors: their roles in the maintenance of skeletal muscle homeostasis. *Cellular and molecular life sciences : CMLS*. 2014;71(9):1657-71.

34. Semenza GL. Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. *Experimental physiology*. 2006;91(5):803-6.

35. Slysz J, Stultz J, Burr JF. The efficacy of blood flow restricted exercise: A systematic review & meta-analysis. *J Sci Med Sport*. 2015.

36. Steinberg GR, Kemp BE. AMPK in Health and Disease. *Physiological reviews*. 2009;89(3):1025-78.

37. Taylor CW, Ingham SA, Ferguson RA. Acute and chronic effect of sprint interval training combined with postexercise blood-flow restriction in trained individuals. *Experimental physiology*. 2016;101(1):143-54.

38. Thom R, Rowe GC, Jang C, Safdar A, Arany Z. Hypoxic induction of vascular endothelial growth factor (VEGF) and angiogenesis in muscle by truncated peroxisome proliferator-activated receptor gamma coactivator (PGC)-1alpha. *J Biol Chem*. 2014;289(13):8810-7.

39. Wilkinson SB, Phillips SM, Atherton PJ et al. Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. *J Physiol*. 2008;586(Pt 15):3701-17.

40. Ydfors M, Fischer H, Mascher H, Blomstrand E, Norrbom J, Gustafsson T. The truncated splice variants, NT-PGC-1alpha and PGC-1alpha4, increase with both endurance and resistance exercise in human skeletal muscle. *Physiol Rep*. 2013;1(6):e00140.

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).

Figure 5. (A) Adenosine Monophosphate-Activated Protein (AMPK)^{Thr172} and (B) p53^{Ser15} 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).