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

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

4

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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

76 modes (17). The transcriptional co-activator Peroxisome proliferator-activated receptor
77 gamma (PPARG) coactivator 1 alpha (PGC-1α) is a 'master regulator' of many
78 endurance exercise-induced adaptations by virtue of its central role in promoting
79 mitochondrial biogenesis, angiogenesis, and inflammatory proteins (17). Transcription
80 of the PGC-1α gene has been shown to be under the control of several promoter regions
81 with activation of the alternative PGC-1α1 promoter resulting in the transcription of
82 three additional isoforms: PGC-1α2, -α3 and-α4. Ruas and colleagues (32) recently
83 demonstrated a preferential increase in the PGC1-α4 isoform following resistance
84 exercise in human skeletal muscle. However, little is known about the regulation of the
85 α2 and α3 isoforms and, to date, no studies have investigated the expression of all four
86 PGC-1α isoforms to diverse contractile stimuli such as resistance and endurance
87 exercise, or following BFR, in humans. Accordingly, the aim of the present study was
88 to compare the acute molecular responses mediated by the different PGC-1α isoforms
89 following low intensity endurance exercise (BFR-EE), resistance exercise (RE) and
90 moderate endurance exercise (EE). As BFR-EE can promote both endurance capacity
91 and muscle hypertrophy responses, we hypothesised EE and RE would selectively
92 increase the expression of the PGC-1α1 and α4 isoforms, respectively. In contrast, we
93 hypothesized that BFR-EE would upregulate a molecular signature involving the
94 increase of both isoforms and their respective anabolic and mitochondrial gene targets.
95
96 **METHODS**
97 *Subjects*
98 Nine untrained, healthy male subjects [age 22.4 ± 3.0 yr, body mass (BM) 73.5 ±
99 9.7 kg, height 1.79 ± 0.05 m, maximal oxygen uptake test (VO_{2peak}) 36.8 ± 4.8 $mLlkg^{-}$
100 $^{1}\cdot min^{-1}$, leg press one repetition maximum (1-RM) 266 ± 66 kg; values are mean ± SD]

101 voluntarily participated in this study. The experimental procedures and possible risks
102 associated with the study were explained to all subjects, who provided written informed
103 consent before participation. The study was approved by the local University's Ethics
104 Committee and conducted in conformity with the policy statement regarding the use of
105 human subjects according to the latest revision of the *Declaration of Helsinki*.
106
107 *Experimental Design*
108 The study employed a randomized counter-balanced, cross-over design in which
109 each subject completed a bout of either resistance exercise (RE), endurance cycling
110 exercise (EE) or low-intensity cycling exercise combined with blood flow restriction
111 (BFR-EE). Two weeks prior to the first exercise session, a resting muscle biopsy was
112 obtained before participants underwent VO_{2peak} and one-repetition maximum (1-RM)
113 testing, and exercise familiarization. Exercise trials were separated by a one-week
114 recovery period during which time subjects maintained their habitual diet and physical
115 activity patterns.
116
117 *Preliminary Testing*
118 *VO_{2peak}*. Participants performed a maximum graded exercise test on a cycle ergometer
119 with electromagnetic braking (Quinton modelo: Corival 400, Lode BV, Groningen,
120 Netherlands) based on a protocol used in previously published paper that investigated
121 BFR-EE (1). Briefly, after resting on the bike for 5 min, participants commenced the
122 incremental test protocol. Briefly, subjects commenced cycling at an initial load of 50
123 W for 1 min and the workload was increased by 15 W/min until a workload of 200 W
124 was reached, after which further increases were 10 W/min increments. The test
125 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

151 consumption for a minimum of 48 h. Subjects were provided with standardized
152 prepacked meals that consisted of 3 g carbohydrate/kg body mass (BM), 0.5 g
153 protein/kg BM, and 0.3 g fat/kg body mass consumed as the final caloric intake the
154 evening before reporting for an experimental trial.

155

156 Experimental Testing Sessions

157 On the morning of an experimental trial, subjects reported to the laboratory after a ~10-
158 h overnight fast. After resting in the supine position for ~15 min and under local
159 anaesthesia (2–3 mL of 1% Xylocaine), a resting biopsy was obtained from the *vastus*
160 *lateralis* using a 5-mm Bergstrom needle modified with suction (7). Approximately 100
161 mg of muscle was removed, dissected free from blood and connective tissue and snap
162 frozen in liquid nitrogen before being stored at – 80°C until subsequent analyses. Due to
163 ethical constraints regarding the total number of muscle biopsies allowed, this single
164 resting biopsy was used as a basal control for all subsequent exercise trials. Two weeks
165 later participants returned to the laboratory having (after the same pre-trial diet and
166 exercise control) to undertake the first of three randomly assigned exercise sessions
167 (described below). Each exercise trial was separated by a one week wash out. Following
168 the completion of each exercise session, subjects rested for 180 min after which time a
169 muscle biopsy was obtained. Subsequent incisions were performed 3 cm proximal to
170 each other. Blood samples were collected before each exercise session and immediately,
171 1, 2 hr and 3 hr post exercise. Blood samples were immediately placed in microtubes
172 containing 1% sodium fluoride and then centrifuged at 3000 rpm for 5 min to separate
173 the plasma before being aliquoted and frozen in liquid nitrogen and stored at -80°C.

174

175 *Resistance Exercise (RE)*

176 After a standardized warm-up on a cycle ergometer consisting of 5 min light
177 cycling at 25 W, subjects performed 4 sets of 10 repetitions leg press exercise (45° leg
178 press machine; G3-PL70; Matrix) at 70% of 1-RM. Each set was separated by a 1 min
179 recovery period during which time subjects remained seated on the leg press machine.
180 Complete concentric/eccentric movements were performed with 90° of range of motion
181 and strong verbal encouragement was provided during each set. The volume and
182 intensity of this session was based on the recommendations of American College Sports
183 Medicine (ACSM) (3). All participants completed every repetition from each respective
184 set.

185

186 *Endurance Exercise (EE)*

187 Following a standardized warm up (described previously), subjects performed
188 30 min of continuous cycling at a power output that elicited ~at 70% of individual
189 VO_{2peak}. Subjects were fan-cooled and provided visual feedback for pedal frequency,
190 power output, and elapsed time were provided to subjects. The volume and intensity of
191 this session were based on the recommendations of ACSM (4). All participants
192 completed the full 30 min session.

193

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

195 Subjects performed 15 min continuous cycling with a cuff strapped over the
196 thigh at a power output that elicited at 40% of VO_{2peak}, as previously reported (1). An
197 18-cm wide cuff was placed on the proximal portion of the thigh (inguinal fold region)
198 over the tibial artery and once in position, was inflated until an absence of auditory
199 blood pulse detected through auscultation with a vascular Doppler probe (DV-600;
200 Marted, São Paulo, Brazil). Pressure was then slowly released until the first arterial

201 pulse was detected which was considered the systolic pressure at the tibial artery. Cuff
202 pressure was set at 80% of the maximum tibial arterial pressure and the cuff was
203 inflated through-out the entire exercise session (22).

204

205 ***Analytical Procedures***

206 *Blood Lactate*

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

210

211 *RNA Extraction and Quantification*

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

218

219 *Reverse Transcription*

220 First-strand complementary DNA (cDNA) synthesis was performed using
221 commercially available TaqMan Reverse Transcription Reagents (Invitrogen,
222 Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative
223 control samples were reverse transcribed to cDNA in a single run from the same reverse
224 transcription master mix. Serial dilutions of a template human skeletal muscle RNA
225 (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription

226 and for calculation of a standard curve for real-time quantitative polymerase chain
227 reaction (RT-PCR).
228
229 *Real-Time PCR*
230 Quantification (in duplicate) of mRNA was performed using a CFX96 Touch™
231 Real-Time PCR Detection System (Bio Rad, California, USA). Taqman-FAM-labelled
232 primer/probes for MuRF-1 (Cat No. Hs00822397_m1), COXIV (Cat No.
233 Hs00971639_m1), IL-6 (Cat No. Hs00985639_m1), PGC-1α (Cat No.
234 Hs01016719_m1), HIF-1α (Cat No. Hs00153153_m1), Myostatin (Hs00976237_m1),
235 IGF-1 (Hs01547656_m1) and VEGF (Cat No. Hs00900055_m1) were used in a final
236 reaction volume of 20 µL. PCR treatments were 2 min at 50 ºC for UNG activation, 10
237 min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60s. Glyceraldehyde-3-
238 phosphate dehydrogenase (GAPDH) (Cat No Hs02758991_g1) was used as a
239 housekeeping gene and was stably expressed between exercise interventions (data not
240 shown). The relative amounts of mRNAs were calculated using the relative
241 quantification (ΔΔCT) method (24). All Taqman-based PCR experiments were
242 performed in the Centre for Exercise and Nutrition laboratory at the Australian Catholic
243 University.
244
245 *Quantification of PGC-1α isoforms*
246 RNA was extracted from a separate piece of snap frozen muscle (~20 mg) using TRIzol
247 (Invitrogen) and purified using QIAGEN RNeasy mini-columns. Reverse transcription
248 was performed using a High Capacity cDNA Reverse Transcription kit (Applied Bio-
249 systems). Real-Time Quantitative PCR was carried out in a SYBR Green ER PCR
250 Master Mix (Invitrogen)/ 384-well format using an ABI PRISM 7900HT (Applied

251 Biosystems). Relative mRNA levels were calculated using the comparative CT method
252 and normalized to cyclophilin mRNA. Primer sequences are as follows: Cyclophilin
253 (forward: GGAGATGGCACAGGAGGAA; reverse: GCCCGTAGTGC TTCAGTTT),
254 PGC1α1 (forward: ATG GAG TGA CAT CGA GTG TGC T; reverse: GAG TCC ACC
255 CAG AAA GCT GT), PGC1α2 (forward: AGT CCA CCC AGA AAG CTG TCT;
256 reverse: ATG AAT GAC ACA CAT GTT GGG), PGC1α3 (forward: CTG CAC CTA
257 GGA GGC TTT ATG C; reverse: CAA TCC ACC CAG AAA GCT GTC T), and
258 PGC1α4 (forward: TCA CAC CAA ACC CAC AGA GA; reverse: CTG GAA GAT
259 ATG GCA CAT). All SYBR Green-based PCR experiments were performed in the
260 Department of Cell Biology laboratory at the Dana-Farber Cancer Institute, Harvard
261 Medical School (USA).
262
263 *Western Blots*
264 Approximately 30 mg of muscle was homogenized in buffer containing 50 mM
265 Tris·HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM
266 NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10% µg/ml trypsin inhibitor, 2 µg/ml
267 aprotinin, 1mM benzamidine, and 1 mM PMSF. After determination of protein
268 concentration (Pierce, Rockford, IL), lysate was resuspended in Laemmli sample buffer.
269 Lysate was then re-suspended in Laemmli sample buffer with 40 µg of protein loaded
270 onto 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (Bio Rad, California, USA). Post
271 electrophoresis gels were activated according to the manufacturer's details (Chemidoc,
272 Bio-Rad, Gladesville, Australia) and then transferred to polyvinylidine fluoride (PVDF)
273 membranes. After transfer, a Stain-Free image of the PVDF membranes for total protein
274 normalization was obtained before membranes were rinsed briefly in distilled water and
275 blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and

276 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C.
277 Membranes were incubated with secondary antibody (1:2,000), and proteins were
278 detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK;
279 Pierce Biotechnology, Rockford, IL) and quantified by densitometry. All sample time
280 points for each subject were run on the same gel. Polyclonal anti-phospho-mTOR^{Ser2448}
281 (no. 2971), -p70 S6K^{Thr389} (no. 9206), - adenosine monophosphate kinase (AMPK)^{Thr172}
282 (no. 2531), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) 4E-
283 BP1$^{Thr37/46}$ (no. 9459), eEF2 eukaryotic translation elongation factor 2 (eEF2) eEF2^{Thr56}
284 (no. 2331) and p53^{Ser15} (no. 9284) were purchased from Cell Signaling Technology
285 (Danvers, MA, USA). Volume density of each target protein band was normalized to
286 the total protein loaded into each lane using stain-free technology (15) with data
287 expressed in arbitrary units. Due to low sample availability, phosphorylated proteins
288 were unable to be normalized to their respective total protein content and were therefore
289 also normalized to stain-free protein levels.
290
291 **Statistical analysis**
292 Statistical analysis was performed using SAS version 9.3 for Windows (SAS
293 Institute Inc., Cary, NC, USA). Data normality and variance equality were assessed
294 through the Shapiro-Wilk and Levene tests. One-way ANOVA with repeated measures
295 (factor: condition) was performed for gene and protein expression analyses. A mixed
296 model ANOVA, assuming group and time as fixed factors and subjects as a random
297 factor, was performed for blood lactate data. Tukey post hoc analysis was used for
298 multiple comparison purposes when significant F-values were found. The significance
299 level was set at $P \leq 0.05$. Data are presented as Mean ± Standard Deviation (SD).
300

301 **Results**

302 **mRNA expression**

303 **Total PGC1-α and isoforms**

304 Total PGC-1α mRNA (Figure 1A) increased with EE above rest (P<0.0001), RE
305 (P=0.0013) and BFR-EE (P>0.0001). There was a significant increase in PGC-1α1
306 mRNA with EE above rest (P=0.0450), RE (P=0.0069) and BFR-EE (P=0.0349)
307 (Figure 1B). There was also a significant increase in PGC-1α2 mRNA (Figure 1C) with
308 EE above rest (P<0.0001), RE (P=0.0003) and BFR-EE (P<0.0001). PGC-1α3 mRNA
309 (Figure 1D) increased with EE above rest (P=0.0389). There was also increases PGC-
310 1α4 mRNA (Figure 1E) with EE above rest (P=0.0035), RE (P=0.0469) and BFR-EE
311 (P=0.0140).

312

313 **Figure 1 here**

314

315 **VEGF, COXIV, HIF-1a**

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

320

321 **Figure 2 here**

322

323 **IL-6 – IGF-1 - Myostatin - MurRF1**

324 IL-6, IGF-1 and Myostatin mRNA expression were unchanged post-exercise
325 (Figure 3 A, B, C). There was a post-exercise increase in MuRF1 mRNA abundance

326 with EE above Rest (P=0.0003), RE (P=0.0256) and BFR-EE (P=0.0007) (Figure 3D).

327

328 **Figure 3 here**

329

330 **Cell Signaling**

331 **mTOR -p70S6K -4E-BP1 -eEF2**

332 There were no changes in $mTOR^{Ser2448}$, $p70S6K^{Thr389}$, 4E-$BP1^{Thr37/46}$or $eEF2^{Thr56}$

333 phosphorylation post-exercise or between exercise groups (Figure 4).

334

335 **Figure 4 here**

336

337 **AMPK -p53**

338 $AMPK^{Thr156}$and $p53^{Ser15}$ phosphorylation were unchanged post-exercise (Figure

339 5).

340

341 **Figure 5 here**

342

343 **Plasma lactate concentration**

344 Lactate concentration increased above rest immediately post-exercise for all

345 interventions (P<.0001 for all comparisons; Table 1). Lactate concentration remained

346 elevated at 1 h, 2 h and 3 h post-exercise for EE and RE, and 1 h and 2 h for BFR-EE

347 (P<.0001 for all comparisons).

348

349 **Table 1 here**

350

351 **Discussion**

352 Low intensity (<50% of VO_{2peak}) endurance training with blood flow restriction

353 has been shown to concomitantly promote isometric muscle strength, muscle CSA and

354 VO_{2peak} (1, 2). While these enhanced adaptation responses are considerably lower in

355 magnitude compared to conventional resistance or endurance exercise performed

356 without any blood flow restriction, the underlying molecular mechanisms mediating

357 these responses remain largely undefined. For the first time we report that low intensity

358 endurance cycling exercise performed with blood flow restriction failed to increase

359 PGC-1α expression to that commonly observed with 'conventional' endurance exercise.

360 Moreover, we show isoform-specific post-exercise increases in the α4 isoform along

361 with Hif-1α and VEGF mRNA expression following higher intensity endurance

362 exercise without blood flow restriction. Taken collectively, our novel findings suggest

363 that cycle exercise undertaken with blood flow restriction is unable to provoke the

364 perturbations to cellular homeostasis necessary to induce activation of the cell signaling

365 events regulating mitochondrial biogenesis and angiogenesis that take place with higher

366 intensity endurance exercise without blood flow restriction.

367

368 A growing body of evidence suggests that exercise undertaken with blood flow

369 restriction can enhance exercise adaptation. A recent meta-analysis reported both low

370 load/intensity resistance (20–30% 1 RM) and aerobic walking exercise performed with

371 blood flow restriction can induce increases in muscle strength and hypertrophy,

372 although with smaller gains compared to high intensity resistance exercise alone (35).

373 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

399 substrate metabolism that can translocate to the nucleus upon activation and induce
400 PGC-1α expression (17). Phosphorylation of either of these protein targets was
401 unaltered post-exercise suggesting other molecular markers and/or physiological
402 mechanisms may be responsible for the upregulation of PGC-1α with high intensity
403 endurance exercise. One plausible explanation for these discrepant findings may be the
404 level of glycogen utilization between exercise sessions in our untrained subjects. We
405 (10) and others (6, 31) have shown greater post-exercise PGC-1α expression with low-
406 compared to normal or high glycogen concentration and although we did not measure
407 muscle glycogen use in the current study due to limited muscle tissue availability, the
408 longer duration and higher intensity exercise bout is likely to have induced greater
409 glycogen depletion compared to the endurance exercise session performed with blood
410 flow restriction.
411
412 Another possible explanation for the discrepancy in PGC-1α1 expression between the
413 two endurance-based exercise bouts is the large differences in estimated energy
414 expenditure. Exercise energy expenditure after BFR-EE was ~4 fold less compared to
415 the EE protocol with total energy expenditure positively associated with PGC-1α
416 expression ($r=0.73$, $P=0.039$). Increased PGC-1α mRNA expression has been observed
417 after 30 min running compared to bouts of 20 and 10 min (37). Thus, total exercise-
418 induced energy expenditure may be an overriding determinant of PGC-1α expression
419 responses post-exercise.
420
421 Low intensity endurance exercise with BFR was also unable to induce the expression of
422 PGC-1α4 compared to higher intensity endurance exercise without blood flow
423 restriction. The PGC-1α4 isoform has been proposed to promote muscle hypertrophy by

424 inducing IGF-1 expression and reducing the expression of myostatin, a negative
425 regulator of muscle growth (32). The increase in PGC-1α4 mRNA expression with EE
426 in the current study was mirrored by a small, non-significant, increase and decrease in
427 IGF1 and myostatin expression, respectively. Ruas and colleagues were the first to
428 show a selective increase in PGC-1α4 expression (concomitant with decreased
429 myostatin abundance) with resistance compared to endurance exercise in human skeletal
430 muscle (32). However, this expression pattern was observed following 8 weeks whole-
431 body resistance training. Thus, a limitation of our study is that we only incorporated a
432 single bout of isolated leg press suggesting longer training programs/ exercise stimulus
433 may be required to induce this selective PGC-1α4 response. Nonetheless, another recent
434 publication reported increased truncated and non-truncated PGC-1a transcripts from
435 both alternative and proximal promoter sites 2 hours following an acute bout of
436 resistance exercise that incorporated the same volume and intensity as our study (40).
437 This indicates the resistance exercise bout performed in our study was likely sufficient
438 to induce the appropriate signal to increase the expression of this isoform however
439 potential differences in post-exercise biopsy timing between this study and ours (2 h vs.
440 3 h) may explain why we did not observe this increase with resistance exercise.

441

442 Increased PGC-1α4 and VEGF expression has also been reported in primary myotubes
443 treated under hypoxic conditions suggesting low oxygen conditions to be favorable for
444 the activation of this isoform (38). In the current study, the transcription factor Hif-1α, a
445 key regulator of angiogenesis in situations of hypoxia (34), was unchanged following
446 BFR-EE, while RE and EE induced 2-fold higher post-exercise changes in lactate
447 compared to BFR-EE. While it is possible a greater metabolic and hypoxic stimulus
448 may be required to increase PGC-1α4 signaling, others have reported unchanged blood

449 lactate following aerobic-based exercise with blood flow restriction (26). Moreover, the
450 same occlusion protocol (15 min cycle at 40% VO_{2peak}) has been shown to improve
451 muscle volume and VO_{2peak}, during a chronic training intervention (1). Thus, it is
452 possible chronic exposure to this occlusion stimulus may be required to elicit increases
453 in PGC-1α4 expression. As this is the first study to investigate changes in Hif-1α
454 following endurance cycling exercise with BFR it is difficult to compare our results to
455 those of previous investigations incorporating resistance exercise and BFR. However,
456 we speculate that when performed with blood flow restriction, the lower contractile
457 intensity associated with 'conventional' endurance compared to resistance (or sprint)
458 exercise, provides adequate blood flow to the exercising musculature and adjoining
459 capillary beds in order to prevent tissue de-oxygenation. Further studies comparing
460 different low intensity endurance exercise protocols with resistance exercise that
461 incorporate blood flow restriction are required to corroborate this hypothesis.
462
463 Another novel finding from the current study was the post-exercise increases in the
464 PGC-1α2 and 3 isoforms. Similar to the α1 and α4 isoforms, both PGC-1α2 and α3
465 increased above rest with higher intensity endurance exercise and were significantly
466 elevated compared to resistance exercise. Both isoforms are expressed in skeletal
467 muscle and brown adipose tissue although little is known about the regulatory targets of
468 these isoforms and their capacity to mediate exercise adaptation responses (27). Based
469 on the elevated response following endurance compared to resistance exercise, we
470 propose these isoforms to mediate physiological processes related to mitochondrial
471 biogenesis and substrate metabolism.
472

473 Considering low load endurance exercise with BFR can increase muscle strength and
474 hypertrophy (35), we also investigated markers of translation initiation, elongation and
475 muscle proteolysis. Previous studies have reported increases in mTOR and p70S6K
476 phosphorylation that have formed the basis for enhanced rates of muscle protein
477 synthesis following resistance exercise with blood flow restriction (13, 14).
478 Nonetheless, the phosphorylation status of these proteins as well as 4E-BP1 and eEF2
479 were unchanged 3 h post-exercise in the current study. This is in agreement with the
480 results of Ozaki and colleagues (28) who observed no changes in Akt, mTOR or
481 p70S6K phosphorylation following 20 min treadmill walking performed with blood
482 flow restriction despite a higher intensity exercise bout (55% VO_{2peak}) compared to our
483 protocol. While our study design was somewhat limited by only having the single post-
484 exercise biopsy (9), this sampling time-point was specifically chosen based on previous
485 studies showing significant, and in some cases maximal, increases in PGC-1α mRNA
486 expression in response to an exercise challenge (5, 23). Future studies investigating
487 endurance exercise undertaken with BFR-EE should include a time-course of signaling
488 responses in order to determine the optimal 'window' for muscle sampling in
489 subsequent investigations.
490
491 Several other factors including the width and pressure of cuff used during BFR must
492 also be considered. Previous studies have reported smaller increases in muscle CSA
493 when lower body resistance training is undertaken with BFR (compared to no BFR) at
494 the site of the cuff (12, 20). While this indicates a narrow cuff may be advantageous for
495 promoting anabolic adaptation responses due to compressing less muscle tissue, a recent
496 study comparing the effects of a wide versus narrow cuff reported similar increases in
497 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.

Acknowledgments

The authors would like to express gratitude for the FAPESP (2014/00985-0) and FAEPEX for financial support. The writing of this publication was also supported by a Collaborative Research Networks (CRN) grant awarded to J. A. Hawley (2013000443). The authors declare no declare no conflicts of interest. The results of the present study do not constitute endorsement by ACSM.

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671 **Figure 1.** (A) Total Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-
672 1α), (B) Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α1), (C)
673 Peroxisome proliferator-activated receptor-γ coactivator 1α2 (PGC-1α2), (D)
674 Peroxisome proliferator-activated receptor-γ coactivator 1α3 (PGC-1α3) and (E)
675 Peroxisome proliferator-activated receptor-γ coactivator 1α4 (PGC-1α4) mRNA
676 abundance at rest and 3 h post-exercise recovery following endurance exercise (EE),
677 resistance exercise (RE) or low-intensity associated with blood flow restriction (BFR-
678 EE). Values are expressed relative to GAPDH and presented in arbitrary units (mean ±
679 SD, n=9). a= Significant different from Rest ($P \leq 0.05$); b= Significant different from
680 HI-RT ($P \leq 0.05$); c= Significant different from BFR-EE ($P \leq 0.05$).
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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 ($P \leq 0.05$).

721 **Figure 3.** (A) Interleukin 6 (IL-6), (B) Insulin-like growth factor 1(IGF-1), (C) Muscle
722 RING finger 1 (MURF1) and (D) Myostatin mRNA abundance at rest and 3 h post-
723 exercise recovery following endurance exercise (EE), resistance exercise (RE) or low-
724 intensity associated with blood flow restriction (BFR-EE). Values are expressed relative
725 to GAPDH and presented in arbitrary units (mean ± SD, n=9). a= Significant different
726 from Rest (P≤ 0.05); b= Significant different from HI-RT (P≤ 0.05); c= Significant
727 different from BFR-EE (P≤ 0.05).
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740 **Figure 4.** (A) Mechanistic target of rapamycin (mTOR)^{Ser2448} (B) p70S6K^{Thr389} (C)
741 eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)$^{Thr37/46}$ eukaryotic
742 elongation factor 2 (eEF2)^{Thr56} phosphorylation in skeletal muscle at rest and after 3 h
743 post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or
744 low-intensity associated with blood flow restriction (BFR-EE). Values are normalized
745 to total protein loaded determined by stain free technology in arbitrary units (mean ±
746 SD, n=9).

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

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