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 Acute low-intensity cycling with blood-flow restriction has no effect on metabolic signaling in human skeletal muscle compared to traditional exercise

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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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26 **ABSTRACT**

27 Introduction: Exercise performed with blood flow restriction simultaneously enhances

28 the acute responses to both myogenic and mitochondrial pathways with roles in training

29 adaptation. Methods: We investigated isoform-specific gene expression of the

30 peroxisome proliferator-activated receptor gamma (PPARG) coactivator 1 and selected

31 target genes and proteins regulating skeletal muscle training adaptation. Methods: 9

32 healthy, untrained males participated of a randomized counter-balanced, cross-over

33 design in which each subject completed a bout of low-intensity endurance exercise

34 performed with blood flow restriction (15 min cycling at 40% of VO_{2peak}, BFR-EE),

35 endurance exercise (30 min cycling at 70% of VO_{2peak}, EE) or resistance exercise (4 x

36 10 repetitions of leg press at 70% of 1-repetition maximum, RE), followed by one

37 biopsy (vastus lateralis) 3 hr after each boat. All exercise sessions were separate by one

38 week. As control a single resting muscle biopsy was obtained two weeks before the first

39 exercise trial (rest). Results: Total PGC-1α mRNA abundance, along with all four

40 isoforms, increased above rest with EE only ($P<0.05$) being higher than BFR-EE

41 ($P<0.05$). PGC-1α1, 2 and 4 were higher after EE compared to RE ($P<0.05$). EE also

42 increased VEGF, Hif-1α and MuRF-1 mRNA abundance above rest ($P<0.05$) while

43 COXIV mRNA expression increased with EE compared to BFR-EE ($P<0.05$).

44 Conclusion: The attenuated expression of all four PGC-1α isoforms when endurance

45 exercise is performed with blood flow restriction suggests this type of exercise provides

46 an insufficient stimulus to activate the signaling pathways governing mitochondrial and

47 angiogenesis responses observed with moderate- to high intensity endurance exercise.

48

49 Key words: mitochondrial biogenesis; cell signalling; skeletal muscle; adaptation;

50 angiogenesis; high intensity exercise

51

52

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).

62

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.

74

75   The cellular mechanisms mediating adaptation responses to exercise are

76   complex involving the cross talk of several intracellular signaling systems that

77   ultimately form the basis for specific phenotypic responses with divergent contractile

78   modes (17). The transcriptional co-activator Peroxisome proliferator-activated receptor

79   gamma (PPARG) coactivator 1 alpha (PGC-1α) is a 'master regulator' of many

80   endurance exercise-induced adaptations by virtue of its central role in promoting

81   mitochondrial biogenesis, angiogenesis, and inflammatory proteins (20). Transcription

82   of the PGC-1α gene has been shown to be under the control of several promoter regions

83   with activation of the alternative PGC-1α1 promoter resulting in the transcription of

84   three additional isoforms: PGC-1α2, -α3 and-α4. Ruas and colleagues (30) recently

85   demonstrated a preferential increase in the PGC1-α4 isoform following resistance

86   exercise in human skeletal muscle. However, little is known about the regulation of the

87   α2 and α3 isoforms and, to date, no studies have investigated the expression of all four

88   PGC-1α isoforms to diverse contractile stimuli such as resistance and endurance

89   exercise in humans. Accordingly, the aim of the present study was to compare the acute

90   molecular responses mediated by the different PGC-1α isoforms following low intensity

91   endurance exercise (BFR-EE), resistance exercise (RE) and moderate endurance

92   exercise (EE). As BFR-EE can promote both endurance capacity and muscle

93   hypertrophy responses, we hypothesised EE and RE would selectively increase the

94   expression of the PGC-1α1 and α4 isoforms, respectively. In contrast, we hypothesized

95   that BFR-EE would upregulate a molecular signature involving the increase of both

96   isoforms and their respective anabolic and mitochondrial gene targets.

97

98   **METHODS**

99   Subjects

100   Nine untrained, healthy male subjects [age 22.4 ± 3.0 yr, body mass (BM) 73.5 ±

101   9.7 kg, height 1.79 ± 0.05 m, maximal oxygen uptake test (VO_{2peak}) 36.8 ± 4.8 mLlkg$^-$

102    $^1 \cdot$min^{-1}, leg press one repetition maximum (1-RM) 266 ± 66 kg; values are mean ± SD]

103   voluntarily participated in this study. The experimental procedures and possible risks

104   associated with the study were explained to all subjects, who provided written informed

105   consent before participation. The study was approved by the local University's Ethics

106   Committee and conducted in conformity with the policy statement regarding the use of

107   human subjects according to the latest revision of the Declaration of Helsinki.

108

109   Experimental Design

110   The study employed a randomized counter-balanced, cross-over design in which

111   each subject completed a bout of either resistance exercise (RE), endurance cycling

112   exercise (EE) or low-intensity cycling exercise combined with blood flow restriction

113   (BFR-EE). Two weeks prior to the first exercise session, a resting muscle biopsy was

114   obtained before participants underwent VO_{2peak} and one-repetition maximum (1-RM)

115   testing, and exercise familiarization. Exercise trials were separated by a one-week

116   recovery period during which time subjects maintained their habitual diet and physical

117   activity patterns.

118

119   Preliminary Testing

120   VO_{2peak}. Participants performed a maximum graded exercise test on a cycle

121   ergometer with electromagnetic braking (Quinton modelo: Corival 400, Lode BV,

122   Groningen, Netherlands). After resting on the bike for 5 min, participants commenced

123   the incremental test protocol. Briefly, subjects commenced cycling at an initial load of

124   50 W for 1 min and the workload was increased by 15 W/min until a workload of 200

125   W was reached, after which further increases were 10 W/min increments. The test

126   continued until voluntary exhaustion, defined by two of the three following criteria:

127   VO_{2peak} plateau (< 2.1 mL.kg^{-1}.min^{-1} of variation), > 1.10 respiratory exchange ratio,

128   and/or heart rate higher than 90% of maximum estimated from age (19). Gas exchange

129   data were collected continuously using an automated breath-by-breath metabolic system

130   (CPX, Medical Graphics, St. Paul, Minnesota, USA) and the highest oxygen

131   consumption value was defined as the peak oxygen consumption (VO_{2peak}) over any 30

132   sec period.

133

134   Maximal Strength

135   The one-repetition maximum (1-RM) test was performed on a leg press machine

136   (45º leg press, G3-PL70; Matrix, São Paulo, Brazil) as previously described (8). Briefly,

137   participants performed a 5 min warm-up on a cycle ergometer riding at 25 W.

138   Participants then undertook 1 x 10 repetitions at 50% of their estimated 1-RM, followed

139   by 1 x 3 repetitions at 70% of the estimated 1-RM with 1-min rest between sets.

140   Participants then performed a series of single repetitions until the maximum load (1-

141   RM) lifted was established with fully eccentric-concentric movement with 90º range of

142   motion. Repetitions were separated by a 3-min recovery and were used to establish the

143   maximum load/weight that could be moved through the full range of motion once, but

144   not a second time.

145

146   Diet/Exercise Control

147   Before each experimental trial (described subsequently), subjects were instructed

148   to refrain from exercise training and vigorous physical activity, and alcohol and caffeine

149   consumption for a minimum of 48 h. Subjects were provided with standardized

150 prepacked meals that consisted of 3 g carbohydrate/kg body mass (BM), 0.5 g

151 protein/kg BM, and 0.3 g fat/kg body mass consumed as the final caloric intake the

152 evening before reporting for an experimental trial.

153

154 Experimental Testing Sessions

155 On the morning of an experimental trial, subjects reported to the laboratory after a ~10-

156 h overnight fast. After resting in the supine position for ~15 min and under local

157 anaesthesia (2–3 mL of 1% Xylocaine), a resting biopsy was obtained from the vastus

158 lateralis using a 5-mm Bergstrom needle modified with suction (7). Approximately 100

159 mg of muscle was removed, dissected free from blood and connective tissue and snap

160 frozen in liquid nitrogen before being stored at − 80°C until subsequent analyses. Due to

161 ethical constraints regarding the total number of muscle biopsies allowed, this single

162 resting biopsy was used as a basal control for all subsequent exercise trials. Two weeks

163 later participants returned to the laboratory having (after the same pre-trial diet and

164 exercise control) to undertake the first of three randomly assigned exercise sessions

165 (described below). Each exercise trial was separated by a one week wash out. Following

166 the completion of each exercise session, subjects rested for 180 min after which time a

167 muscle biopsy was obtained. Subsequent incisions were performed 3 cm proximal to

168 each other. Blood samples were collected before each exercise session and immediately,

169 1, 2 hr and 3 hr post exercise. Blood samples were immediately placed in microtubes

170 containing 1% sodium fluoride and then centrifuged at 3000 rpm for 5 min to separate

171 the plasma before being aliquoted and frozen in liquid nitrogen and stored at -80°C.

172

173 Resistance Exercise (RE)

174　　　　After a standardized warm-up on a cycle ergometer consisting of 5 min light

175　　cycling at 25 W, subjects performed 4 sets of 10 repetitions leg press exercise (45° leg

176　　press machine; G3-PL70; Matrix) at 70% of 1-RM. Each set was separated by a 1 min

177　　recovery period during which time subjects remained seated on the leg press machine.

178　　Complete concentric/eccentric movements were performed with 90° of range of motion

179　　and strong verbal encouragement was provided during each set.

180

181　　Endurance Exercise (EE)

182　　　　Following a standardized warm up (described previously), subjects performed

183　　30 min of continuous cycling at a power output that elicited ~at 70% of individual

184　　VO_{2peak}. Subjects were fan-cooled and provided visual feedback for pedal frequency,

185　　power output, and elapsed time were provided to subjects.

186

187　　Low Intensity Blood Flow Restriction (BFR-EE)

188　　　　Subjects performed 15 min continuous cycling with a cuff strapped over the

189　　thigh at a power output that elicited at 40% of VO_{2peak}, as previously reported (1). An

190　　18-cm wide cuff was placed on the proximal portion of the thigh (inguinal fold region)

191　　over the tibial artery (38) and once in position, was inflated until an absence of auditory

192　　blood pulse detected through auscultation with a vascular Doppler probe (DV-600;

193　　Marted, São Paulo, Brazil). Pressure was then slowly released until the first arterial

194　　pulse was detected which was considered the systolic pressure at the tibial artery. Cuff

195　　pressure was set at 80% of the maximum tibial arterial pressure and the cuff was

196　　inflated through-out the entire exercise session.

197

198　　**Analytical Procedures**

199   Blood Lactate

200   Plasma lactate concentration was measured on a spectrophotometer (ELx800,

201   Biotek, Winooski, USA) using a commercial kit (Biotecnica, Varginha, Brazil)

202   according to the manufacturer's protocol.

203

204   RNA Extraction and Quantification

205   Approximately 20 mg of skeletal muscle was homogenized in TRIzol with

206   chloroform added to form an aqueous RNA phase. This RNA phase was then

207   precipitated by mixing with ice-cold isopropanol alcohol and the resulting pellet was

208   washed and re-suspended in 40 µl of RNase-free water. Extracted RNA was quantified

209   using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington,

210   USA) by measuring absorbance at 260 nm and 280 nm.

211

212   Reverse Transcription

213   First-strand complementary DNA (cDNA) synthesis was performed using

214   commercially available TaqMan Reverse Transcription Reagents (Invitrogen,

215   Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative

216   control samples were reverse transcribed to cDNA in a single run from the same reverse

217   transcription master mix. Serial dilutions of a template human skeletal muscle RNA

218   (AMBION; Cat No AM7982) was included to ensure efficiency of reverse transcription

219   and for calculation of a standard curve for real-time quantitative polymerase chain

220   reaction (RT-PCR).

221

222   Real-Time PCR

223   Quantification (in duplicate) of mRNA was performed using a CFX96 Touch™

224   Real-Time PCR Detection System (Bio Rad, California, USA). Taqman-FAM-labelled

225   primer/probes for MuRF-1 (Cat No. Hs00822397_m1), COXIV (Cat No.

226   Hs00971639_m1), IL-6 (Cat No. Hs00985639_m1), PGC-1α (Cat No.

227   Hs01016719_m1), HIF-1α (Cat No. Hs00153153_m1), Myostatin (Hs00976237_m1),

228   IGF-1 (Hs01547656_m1) and VEGF (Cat No. Hs00900055_m1) were used in a final

229   reaction volume of 20 µL. PCR treatments were 2 min at 50 ºC for UNG activation, 10

230   min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60s. Glyceraldehyde-3-

231   phosphate dehydrogenase (GAPDH) (Cat No Hs02758991_g1) was used as a

232   housekeeping gene and was stably expressed between exercise interventions (data not

233   shown). The relative amounts of mRNAs were calculated using the relative

234   quantification (ΔΔCT) method (22).

235

236   Quantification of PGC-*1α isoforms*

237   RNA was extracted from a separate piece of snap frozen muscle (~20 mg) using TRIzol

238   (Invitrogen) and purified using QIAGEN RNeasy mini-columns. Reverse transcription

239   was performed using a High Capacity cDNA Reverse Transcription kit (Applied Bio-

240   systems). Real-Time Quantitative PCR was carried out in a SYBR Green ER PCR

241   Master Mix (Invitrogen)/ 384-well format using an ABI PRISM 7900HT (Applied

242   Biosystems). Relative mRNA levels were calculated using the comparative CT method

243   and normalized to cyclophilin mRNA. Primer sequences are as follows: Cyclophilin

244   (forward: GGAGATGGCACAGGAGGAA; reverse: GCCCGTAGTGC TTCAGTTT),

245   PGC1α1 (forward: ATG GAG TGA CAT CGA GTG TGC T; reverse: GAG TCC ACC

246   CAG AAA GCT GT), PGC1α2 (forward: AGT CCA CCC AGA AAG CTG TCT;

247   reverse: ATG AAT GAC ACA CAT GTT GGG), PGC1α3 (forward: CTG CAC CTA

248 GGA GGC TTT ATG C; reverse: CAA TCC ACC CAG AAA GCT GTC T), and

249 PGC1α4 (forward: TCA CAC CAA ACC CAC AGA GA; reverse: CTG GAA GAT

250 ATG GCA CAT).

251

252 Western Blots

253   Approximately 30 mg of muscle was homogenized in buffer containing 50 mM

254 Tris·HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM

255 NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10% μg/ml trypsin inhibitor, 2 μg/ml

256 aprotinin, 1mM benzamidine, and 1 mM PMSF. After determination of protein

257 concentration (Pierce, Rockford, IL), lysate was resuspended in Laemmli sample buffer.

258 Lysate was then re-suspended in Laemmli sample buffer with 40 µg of protein loaded

259 onto 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (Bio Rad, California, USA). Post

260 electrophoresis gels were activated according to the manufacturer's details (Chemidoc,

261 Bio-Rad, Gladesville, Australia) and then transferred to polyvinylidine fluoride (PVDF)

262 membranes. After transfer, a Stain-Free image of the PVDF membranes for total protein

263 normalization was obtained before membranes were rinsed briefly in distilled water and

264 blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and

265 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C.

266 Membranes were incubated with secondary antibody (1:2,000), and proteins were

267 detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK;

268 Pierce Biotechnology, Rockford, IL) and quantified by densitometry. All sample time

269 points for each subject were run on the same gel. Polyclonal anti-phospho-mTOR^{Ser2448}

270 (no. 2971), -p70 S6K^{Thr389} (no. 9206), - adenosine monophosphate kinase (AMPK)^{Thr172}

271 (no. 2531), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) 4E-

272 BP1$^{Thr37/46}$ (no. 9459), eEF2 eukaryotic translation elongation factor 2 (eEF2) eEF2^{Thr56}

273 (no. 2331) and p53^{Ser15} (no. 9284) were purchased from Cell Signaling Technology

274 (Danvers, MA, USA). Volume density of each target protein band was normalized to

275 the total protein loaded into each lane using stain-free technology (15) with data

276 expressed in arbitrary units.

277

278 **Statistical analysis**

279 Statistical analysis was performed using SAS version 9.3 for Windows (SAS

280 Institute Inc., Cary, NC, USA). Data normality and variance equality were assessed

281 through the Shapiro-Wilk and Levene tests. One-way ANOVA with repeated measures

282 (factor: condition) was performed for gene and protein expression analyses. A mixed

283 model ANOVA, assuming group and time as fixed factors and subjects as a random

284 factor, was performed for blood lactate data. Tukey post hoc analysis was used for

285 multiple comparison purposes when significant F-values were found. The significance

286 level was set at $P \leq 0.05$. Data are presented as Mean ± Standard Deviation (SD).

287

288 **Results**

289 **mRNA expression**

290 **Total PGC1-α and isoforms**

291 Total PGC-1α mRNA (Figure 1A) increased with EE above rest (P<0.0001), RE

292 (P=0.0013) and BFR-EE (P>0.0001). There was a significant increase in PGC-1α1

293 mRNA with EE above rest (P=0.0450), RE (P=0.0069) and BFR-EE (P=0.0349)

294 (Figure 1B). There was also a significant increase in PGC-1α2 mRNA (Figure 1C) with

295 EE above rest (P<0.0001), RE (P=0.0003) and BFR-EE (P<0.0001). PGC-1α3 mRNA

296 (Figure 1D) increased with EE above rest (P=0.0389). There was also increases PGC-

297 1α4 mRNA (Figure 1E) with EE above rest (P=0.0035), RE (P=0.0469) and BFR-EE

298   (P=0.0140).

299

300   **Figure 1 here**

301

VEGF, COXIV, HIF-1a

303   There was a significant increase in VEGF mRNA (Figure 2A) with EE above

304   rest (P=0.0180) and RE (P=0.0069). COXIV mRNA expression increased with EE

305   above BFR-EE (P=0.0550) (Figure 2B). There was a significant increase in HIF-1a

306   abundance with EE above Rest (P= 0.0530) (Figure 2C).

307

308   **Figure 2 here**

309

IL-6 – IGF-1 - Myostatin - MurRF1

311   IL-6, IGF-1 and Myostatin mRNA expression were unchanged post-exercise

312   (Figure 3 A, B, C). There was a post-exercise increase in MuRF1 mRNA abundance

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

314

315   **Figure 3 here**

316

Cell Signaling

mTOR -p70S6K -4E-BP1 -eEF2

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

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

321

322   **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.

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

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

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

349 that endurance exercise undertaken with blood flow restriction is unable to provoke the

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

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

352 intensity endurance exercise without blood flow restriction.

353

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

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

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

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

358 although with smaller gains compared to high intensity resistance exercise alone (34).

359 However, little is known about the molecular mechanisms mediating these responses

360 when low intensity endurance exercise is undertaken with blood flow restriction. As

361 such, we compared the expression of key gene and protein targets implicated in a range

362 of exercise adaptation responses such as hypertrophy, mitochondrial biogenesis, muscle

363 proteolysis, substrate metabolism and angiogenesis between BFR-EE, and conventional

364 bouts of RE and EE. We particularly focused on the four different full-length PGC-1α

365 isoforms putatively implicated in anabolic and mitochondrial-related adaptation

366 responses.

367

368 In agreement with previous studies (5, 21, 26), we observed significant increases in total

369 PGC-1α mRNA following continuous endurance exercise performed at 70% of VO_{2peak}.

370 This increase in PGC-1α mRNA was concomitant with greater abundance of VEGF, a

371 target of PGC-1α (36). However, in contrast to our original hypothesis, this response

372 was absent following a bout of low-intensity endurance exercise (40% VO_{2peak})

373 performed with blood flow restriction. In an attempt to identify possible mechanisms

374 responsible for this attenuated PGC-1α response, we investigated IL-6 expression to

375 determine whether an increase in the muscular inflammatory program was implicated in

376 the blunted response. This hypothesis was based on previous data showing an inverse

377 relationship between skeletal muscle PGC-1α and IL-6 expression (16). However, IL-6

378 mRNA expression post-exercise was unchanged in all exercise groups suggesting any

379 acute increase in muscle inflammation caused by BFR-EE was not responsible for the

380 reduced PGC-1α1 expression observed. We also investigated other cellular markers

381 implicated in exercise adaptation responses that can regulate PGC-1α expression.

382 AMPK is an intracellular 'fuel gauge' that can phosphorylate PGC-1α and increase its

383 transcriptional activity (35) while the apoptogenic protein p53 has emerged as another

384 signaling regulator of skeletal muscle exercise-induced mitochondrial biogenesis and

385 substrate metabolism that can translocate to the nucleus upon activation and induce

386 PGC-1α expression (4). Phosphorylation of either of these protein targets was unaltered

387 post-exercise suggesting other molecular markers and/or physiological mechanisms

388 may be responsible for the upregulation of PGC-1α with high intensity endurance

389 exercise. One plausible explanation for these discrepant findings may be the level of

390 glycogen utilization between exercise sessions in our untrained subjects. We (10) and

391 others (6, 29) have shown greater post-exercise PGC-1α expression with low- compared

392 to normal or high glycogen concentration and although we did not measure muscle

393 glycogen use in the current study due to limited muscle tissue availability, the longer

394 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

420 key regulator of angiogenesis in situations of hypoxia (32), was unchanged following

421 BFR-EE, while RE and EE induced 2-fold higher post-exercise changes in lactate

422 compared to BFR-EE. While it is possible a greater metabolic and hypoxic stimulus

423 may be required to increase PGC-1α4 signaling, others have reported unchanged blood

424 lactate following aerobic-based exercise with blood flow restriction (23). Moreover, the

425 same occlusion protocol (15 min cycle at 40% VO_{2peak}) has been shown to improve

426 muscle volume and VO_{2peak}, during a chronic training intervention (1). Thus, it is

427 possible chronic exposure to this occlusion stimulus may be required to elicit increases

428 in PGC-1α4 expression. As this is the first study to investigate changes in Hif-1α

429 following endurance cycling exercise with BFR it is difficult to compare our results to

430 those of previous investigations incorporating resistance exercise and BFR. However,

431 we speculate that when performed with blood flow restriction, the lower contractile

432 intensity associated with 'conventional' endurance compared to resistance (or sprint)

433 exercise, provides adequate blood flow to the exercising musculature and adjoining

434 capillary beds in order to prevent tissue de-oxygenation. Further studies comparing

435 different low intensity endurance exercise protocols with resistance exercise that

436 incorporate blood flow restriction are required to corroborate this hypothesis.

437

438 Another novel finding from the current study was the post-exercise increases in the

439 PGC-1α2 and 3 isoforms. Similar to the α1 and α4 isoforms, both PGC-1α2 and α3

440 increased above rest with higher intensity endurance exercise and were significantly

441 elevated compared to resistance exercise. Both isoforms are expressed in skeletal

442 muscle and brown adipose tissue although little is known about the regulatory targets of

443 these isoforms and their capacity to mediate exercise adaptation responses. Accordingly

444 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.

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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'

470   cleaved myofibril segments for degradation (31). It is unclear whether this increase in

471   expression with high intensity endurance exercise represents general tissue remodeling,

472   particularly considering our participants were untrained and the unaccustomed

473   contractile stimulus, or a greater induction of protein degradation.

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475   In summary, this is the first study to investigate the molecular mechanisms mediating

476   muscle adaptation responses to low intensity endurance cycling exercise with blood

477   flow restriction. The attenuated expression of all four PGC-1α isoforms when endurance

478   exercise is performed with blood flow restriction suggests this type of exercise is unable

479   to induce the appropriate metabolic perturbation capable of activating the cell signaling

480   machinery responsible for mitochondrial biogenesis and angiogenesis responses with

481   moderate-to-high intensity endurance exercise. Longer training programs incorporating

482   endurance exercise with BFR that correlate measurements of these molecular markers

483   with functional adaptation responses such as changes in VO_{2peak} and cycle time to

484   fatigue will yield important information to the efficacy of this training method to

485   enhance training adaptation and subsequently improve health outcomes in populations

486   that may be unable to perform, prolonged exercise.

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488   **Acknowledgment**

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493   **References**

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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≤ 0.05); b= Significant different from HI-RT (P≤ 0.05); c= Significant different from BFR-EE (P≤ 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 ($P \leq 0.05$).

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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≤ 0.05); b= Significant different from HI-RT (P≤ 0.05); c= Significant different from BFR-EE (P≤ 0.05).

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701 **Figure 4.** (A) Mechanistic target of rapamycin (mTOR)^{Ser2448} (B) p70S6K^{Thr389} (C)

702 eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)$^{Thr37/46}$ eukaryotic

703 elongation factor 2 (eEF2)^{Thr56} phosphorylation in skeletal muscle at rest and after 3 h

704 post-exercise recovery following endurance exercise (EE), resistance exercise (RE) or

705 low-intensity associated with blood flow restriction (BFR-EE). Values are normalized

706 to total protein loaded determined by stain free technology in arbitrary units (mean $\pm$

707 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 $\pm$ SD, n=9).

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