Eur J Appl Physiol (2017) 117:2587–2600
DOI 10.1007/s00421-017-3746-2

ORIGINAL ARTICLE

Concomitant external pneumatic compression treatment with consecutive days of high intensity interval training reduces markers of proteolysis

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Received: 1 July 2017 / Accepted: 18 October 2017 / Published online: 26 October 2017

Abstract

Purpose To compare the effects of external pneumatic compression (EPC) and sham when used concurrently with high intensity interval training (HIIT) on performance-related outcomes and recovery-related molecular measures.

Methods Eighteen recreationally endurance-trained male participants (age: 21.6 ± 2.4 years, BMI: 25.7 ± 0.5 kg/m^2, VO_{2peak}: 51.3 ± 0.9 mL/kg/min) were randomized to balanced sham and EPC treatment groups. Three consecutive days of HIIT followed by EPC/sham treatment (Days 2–4) and 3 consecutive days of recovery (Days 5–7) with EPC/sham only on Days 5–6 were employed. Venipuncture, flexibility and pressure-to-pain threshold (PPT) measurements were made throughout. Vastus lateralis muscle was biopsied at PRE (i.e., Day 1), 1-h post-EPC/sham treatment on Day 2 (POST1), and 24-h post-EPC/sham treatment on Day 7 (POST2). 6-km run time trial performance was tested at PRE and POST2.

Results No group × time interaction was observed for flexibility, PPT, or serum measures of creatine kinase (CK), hsCRP, and 8-isoprostane. However, there was a main effect of time for serum CK ($p = 0.005$). Change from PRE in 6-km run times at POST2 were not significantly different between groups. Significant between-groups differences existed for change from PRE in atrogin-1 mRNA ($p = 0.018$) at the POST1 time point (EPC: − 19.7 ± 8.1%, sham: + 7.7 ± 5.9%) and atrogin-1 protein concentration ($p = 0.013$) at the POST2 time point (EPC: − 31.8 ± 7.5%, sham: + 96.0 ± 34.7%). In addition, change from PRE in poly-Ub proteins was significantly different between groups at both the POST1

Communicated by William J. Kraemer.

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(EPC: −26.0 ± 10.3%, sham: + 34.8 ± 28.5%; $p = 0.046$) and POST2 (EPC: − 33.7 ± 17.2%, sham: +21.4 ± 14.9%; $p = 0.037$) time points.

Conclusions EPC when used concurrently with HIIT and in subsequent recovery days reduces skeletal muscle markers of proteolysis.

Keywords Skeletal muscle · Pneumatic compression · Recovery · Proteolysis · Oxidative stress · Endurance exercise · High intensity interval training

Abbreviations

4HNE	4-Hydroxynonenal
Ac	Acetylated
ANOVA	Analysis of variance
APMHR	Age predicted max heart rate
BCA	Bicinchonic acid
BSA	Bovine serum albumin
CK	Creatine kinase
ES	Effect size
Fbl	Fibrillarin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hsCRP	High sensitivity C-reactive protein
EPC	External pneumatic compression
GXT	Graded exercise testing
GPx	Glutathione peroxidase
HIIT	High intensity interval training
IgG	Immunoglobulin G
IκBα	Nuclear factor of kappa B (NF-κB) inhibitor alpha
IL	Interleukin
HR	Heart rate
MCP-1	Monocyte chemoattractant protein-1
MuRF-1	Muscle RING finger 1
NSAIDs	Non-steroidal anti-inflammatories
NF-κB	Nuclear factor of kappa B
PGC-1α	Peroxisome proliferator-activated receptor coactivator 1-alpha
poly-Ub	Poly-ubiquitinated
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPT	Pressure-to-pain threshold
SOD2	Superoxide dismutase 2
TNF-α	Tumor necrosis factorα

Introduction

External pneumatic compression (EPC) is a treatment modality used in both clinical and athletic settings. EPC involves the inflation of cuffs and/or sleeves with air at selected pressures which generally cover the upper or lower extremity limbs. Historically, EPC has been successfully employed in clinical settings to combat lymphedema by increasing lymph transport from limbs and decreasing associated pain from swelling (Muluk et al. 2013; Partsch et al. 2008). However, EPC use for recovery purposes in athletic settings has been increasingly employed and investigated. While some investigations have indicated little to no positive effect of EPC on performance (Cochrane et al. 2013; Martin et al. 2015b, c; Overmayer and Driller 2017) and skeletal muscle glycogen re-synthesis (Keck 2015), several other investigations have reported positive findings which may be pertinent to the recovery-adaptation response when used concurrently with training.

We recently performed an exploratory study examining the effect of EPC on functional and molecular outcomes when used concurrently with consecutive bouts of voluminous resistance exercise (Haun et al. 2017). Briefly, that sham-controlled investigation involved daily EPC use with heavy back squat exercise occurring for three consecutive days followed by three additional days of recovery with EPC treatment only. Similar to the findings of Sands et al. (2014, 2015), we found that EPC treatment attenuated decreases in the pressure-to-pain threshold (PPT) and improved joint range of motion (i.e., flexibility) outcomes compared to sham (Haun et al. 2017). Given that increased muscle soreness is associated with reduced running economy (Braun and Dutto 2003), EPC-mediated attenuation of muscle soreness when used concurrently with endurance training may be beneficial from a metabolic (i.e., reduced anaerobic reliance at relative workloads) and/or risk of injury (Cheung et al. 2003) standpoint. In addition, in the aforementioned EPC and resistance training study, we observed significantly lower levels of surrogates of proteolysis and oxidative stress, total poly-ubiquitinated (poly-Ub) proteins and 4-hydoxynonenal (4HNE), in muscle tissue biopsied from the vastus lateralis with EPC compared to sham (Haun et al. 2017). While the role of oxidative stress in the recovery-adaptation response is controversial (Urso and Clarkson 2003), the implications of a reduction in muscle breakdown are apparent. Finally, we have also previously shown that acute treatment with EPC, independent of exercise, (1) upregulates peroxisome proliferator-activated receptor coactivator 1-alpha (PGC-1α) mRNA in compressed muscle tissue (Kephart et al. 2015) and (2) has a large effect on PGC-1α localization in skeletal muscle cell nuclei (Martin et al. 2016a). PGC-1α is known to be activated by endurance exercise, and is associated with mitochondrial biogenesis and the skeletal muscle endurance phenotype (Baar 2004). Notwithstanding, it remains to be determined if EPC (1) has similar effects in subjects engaged in intense endurance training to those observed when used concurrently with resistance training and (2) has an additive/synergistic effect with known myocellular responses to endurance exercise (e.g., PGC-1).

As a follow-up to our EPC and resistance training investigation, the purpose of this study was to explore the effect(s) of EPC on functional, humoral, and myocellular outcomes when used concurrently with consecutive bouts of intense endurance training. To this end, we chose to investigate the effects of EPC compared to sham when used during and after three consecutive days of high-intensity interval training (HIIT) in recreationally endurance-trained persons. Specifically, we sought to determine the (1) change in functional measures of muscle soreness and flexibility and serum measures of muscle damage, oxidative stress and inflammation across three consecutive days of HIIT and three additional days of recovery with EPC compared to sham; (2) acute effects of a single bout of HIIT followed by EPC on endurance exercise-related skeletal muscle gene expression and protein concentrations compared to a single bout of HIIT followed by sham; and (3) change in 6-km run performance and endurance exercise related skeletal muscle gene expression and protein concentration after three consecutive days of HIIT and three additional days of recovery with daily EPC treatment compared to daily sham treatment.

Methods

Participant characteristics

Prior to initiating this study, the protocol was reviewed and approved by the Auburn University Institutional Review Ethics Committee, and was in compliance with the Helsinki Declaration. All participants gave their informed consent prior to their inclusion in the study. Apparently healthy males ($N = 18$) volunteered to take part in this investigation and completed the Physical Activity Readiness Questionnaire as well as a health history questionnaire to detect potential risk factors that might be aggravated by strenuous physical activity. All participants were considered endurance-trained, participating in ≥ 3 days per week of endurance exercise for at least 3 months.

Experimental protocol

Figure 1 provides an outline of the experimental protocol. For all visits, participants were asked to report following a 4-h fast and at the same time of day ($\pm$ 1-h) to control for metabolic and diurnal variation influence, respectively,

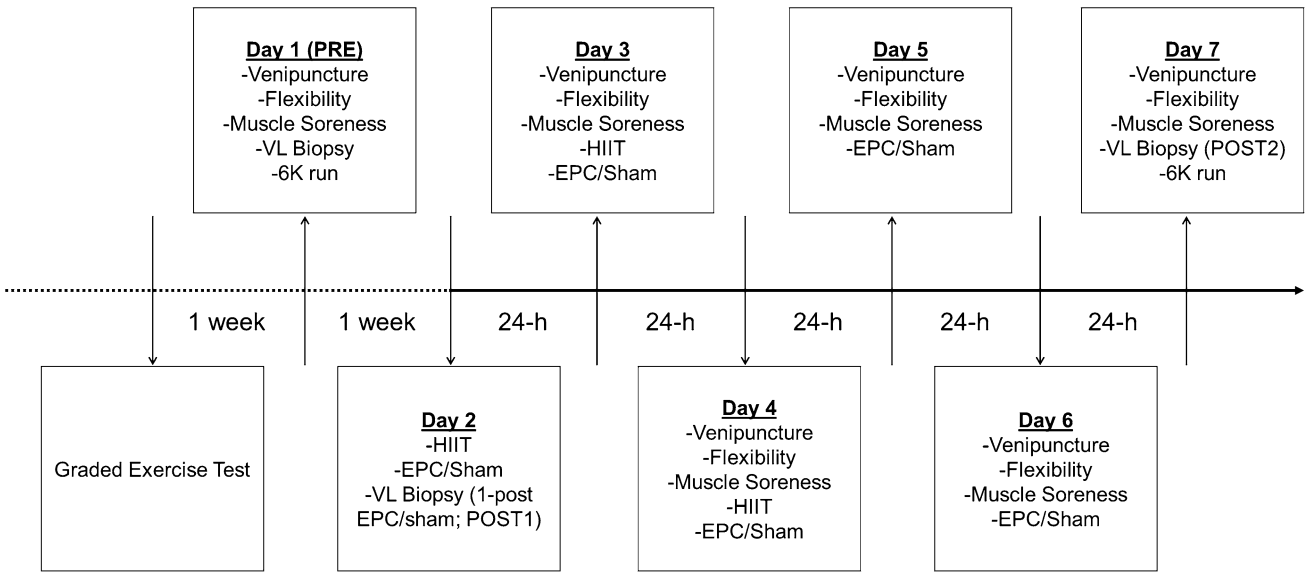

Fig. 1 Time and events for the study protocol. Prior to initiation of study procedures, all participants completed a graded exercise test (GXT) to characterize heart rate responses and VO_{2peak}. 1-week thereafter participants returned for what is termed as Day 1 (i.e., PRE) where the following baseline measurements/collections were performed: venipuncture, right knee range of motion in a modified lung position (i.e., flexibility), pressure-to-pain threshold in the right vastus lateralis (i.e., muscle soreness), biopsy of the left vastus lateralis and 6-km time-trial performance. Participants then reported to the laboratory ~1-week later (i.e., Day 2; POST1) and performed high intensity interval training (HIIT) immediately followed by randomization to either an external pneumatic compression (EPC) or sham treatment group and subsequent 1-h treatment. 1-h following treatment on Day 2, a second biopsy of the left vastus lateralis was performed. On the next two consecutive days (Day 3 and 4) venipuncture was performed followed by flexibility and muscle soreness measures. Thereafter, participants completed the same HIIT followed by respective treatment protocol. On the next two consecutive days (Days 5–6) venipuncture and the muscle soreness and flexibility assessments were performed and were followed by treatment according to group assignment (no HIIT was performed). Finally, on Day 7 (i.e., POST2) participants reported to the lab for venipuncture, muscle soreness and flexibility assessments, a third biopsy of the left vastus lateralis, and a 6-km run time trial performance

on study outcomes. In addition, participants were asked to forgo any strenuous activity for at least 48-h prior to graded exercise testing (GXT) and Day 1 procedures and to refrain from all strenuous activity (except as indicated) throughout the rest of the protocol. Finally, participants were asked to maintain their habitual dietary and sleep habits, not use any "recovery" aids (e.g., ice, topical analgesics, etc.), and to abstain from taking aspirin or non-steroidal anti-inflammatories (NSAIDs) throughout the study protocol, which is described in detail below.

Graded exercise testing

All participants were initially assessed for heart rate (HR) responses and VO_{2peak} during a modified Naughton GXT protocol. Participants were allowed to warm-up by walking on the treadmill (Woodway ELG; Woodway USA, Waukesha, WI USA) at 4.8 km/h (3.0 mph) for 5 min prior to GXT. Immediately after the warm-up was completed, the treadmill incline was set to 1% and speed was set to the participant's self-selected comfortable 10-km run pace. Thereafter, the treadmill grade increased by 2% every 2 min until the participant indicated volitional fatigue. Expired gases during GXT were continuously analyzed using a TrueMax 2400 metabolic measurement system (ParvoMedics, Sandy, UT, USA), averaged in 20-s intervals, and the highest 20-s average for VO_2 was denoted as the VO_{2peak}. Following GXT, participants were dismissed and asked to report back in ~ 1-week.

Day 1 [pre-testing (PRE)]

For the next visit (Day 1 (PRE)], first, venous blood samples were collected in a 5 mL serum separator tube and a 3 mL EDTA tube (BD Vacutainer, Franklin Lakes, NJ, USA) for subsequent analysis of serum and plasma markers, respectively. Then, a baseline (PRE) percutaneous skeletal muscle biopsy from the left vastus lateralis was performed at a site midway between the patella and iliac crest using a 6 mm gauge Bergstrom needle (Product #72-2300506, Millenium Surgical Corp., Narberth, PA USA) with suction and sterile laboratory procedures as described previously (Martin et al. 2016b). Approximately 50 mg of tissue was immediately placed in a 1.7 mL polypropylene tube containing 500 µL of cell lysis buffer (Cell Signaling, Danvers, MA, USA) with pre-added protease and phosphatase inhibitors and processed for protein analyses as described below. Additionally, 10–20 mg of muscle was placed in a 1.7 mL polypropylene tube containing 500 µL of Ribozol (Ameresco, Solon, OH USA) for mRNA analyses as described below, and the remaining tissue was snap-frozen in liquid nitrogen and subsequently stored at − 80 °C. Following biopsy, flexibility was assessed by measuring knee range of motion during a modified kneeling lunge similar to the methods described by MacDonald et al. (2013). Briefly, participants were positioned in the modified lunge position (upright and erect torso, left knee in line with left ankle—perpendicular to floor, right knee in contact with floor behind the torso to the point of stretch—induced discomfort in the right hip). The right hip angle was measured with a HiRes™ plastic 360° goniometer (Baseline®, 12-1000) and this angle was used for subsequent (i.e., Days 3–7) measurements of flexibility. After positioning, the participant's right knee was passively flexed by an investigator until the participant verbally noted the point of discomfort. The right knee angle, in degrees, at this point of stretch was recorded with a goniometer using the lateral malleolus and lateral epicondyle along with the center of the vastus lateralis as landmarks. This procedure was repeated for duplicate measurements after 1 min of passive rest. The observed coefficient of variation for duplicate measures of right knee angle across all days was 3.9%. After flexibility assessment, muscle soreness was measured by applying focal pressure to proximal, medial, and distal targeted areas of the right vastus lateralis using an instrumented algometer (Force Ten FDX, Wagner Instruments, Greenwich, CT, USA). Markings were made for each site with permanent marker and remained visible throughout the duration of the study for each subject. Pressure was applied at a rate of approximately 5 N/s at each site until the subject indicated that the pressure became painful. The point at which the pressure became painful (audibly indicated by participants) was termed the PPT and the value in *N* was recorded. PPT measurements were made sequentially in cycles from the proximal to medial to distal site three times for triplicate measures. The average of the triplicate measures at each site was calculated as the respective site PPT and the average of all measures was calculated as the 3-site PPT. The observed coefficient of variation for triplicate PPT measures across all sites and days was 9.4%. Notably, the digital display of the algometer indicating force of application was blinded to the participants. Finally, to conclude the Day 1 (PRE) visit, participants completed a 6-km run time trial on the treadmill (Woodway ELG) at an incline of 1%. Participants were instructed to complete the 6-km run in the shortest possible time and were allowed to control their own speed during the trial. However, participants and investigators were blinded to all data (e.g., time, speed) except for distance covered.

Days 2–4 (endurance training and treatment bouts)

Approximately 1 week following Day 1 (PRE), participants reported at the same time of day for Day 2 (POST1) procedures. Upon arrival to the laboratory, participants were prepared for and completed HIIT. HIIT was 22.5 min in duration with 15 rounds of running for 45 s and walking for

45 s (1:1 ratio). Briefly, for each interval participants ran on the treadmill (Woodway ELG) at an incline of 1% and a speed resulting in a HR equivalent to 90% of their age-predicted max HR (APMHR; 220-age) for 45 s followed by 45 s of rest (i.e., slow walk on treadmill). Treadmill speed was adjusted as needed for each interval to maintain a HR equivalent to 90% of APMHR ± 3%. HR was monitored continuously using a Polar HR monitor (T31, Polar Electro Inc., Lake Success, NY, USA). Immediately following completion of the exercise bout, participants were randomly assigned to either a 1-h treatment with EPC (NormaTec™ Pro, Newton, MA, USA) or no compression (sham) whereby the participants had the leg sleeve on but not inflated for 1 h. The EPC device, described previously in detail (Haun et al. 2017; Martin et al. 2015c, 2016a), consists of two separate "leg sleeves" which contain five circumferential inflatable chambers (arranged linearly along the limb) encompassing the leg from the feet to the hip/groin. The "leg sleeves" are connected to an automated pneumatic pump at which target inflation pressures for each zone and the duty cycle can be controlled. However, the unit is commercially marketed with pre-programmed defaults for the duty cycle and recommended inflation pressure settings. In this study, we chose to use an inflation protocol consisting of target inflation pressures of ~ 70 mmHg for each chamber and a duty cycle that included 30 s of compression in each zone followed by a 30-s rest period during which all zones are deflated. 1 h following the conclusion of EPC or sham treatments, participants donated a second muscle biopsy from the left vastus lateralis (termed POST1) using techniques and processing procedures described above. The timing of the POST1 biopsy (1-h post EPC, 2-h post HIIT) was chosen (1) in alignment with our previous work on EPC and resistance training (Haun et al. 2017) and (2) based on previous research which demonstrated significant alteration of biopsy derived molecular measures of mRNA, including PGC-1a, following a single EPC treatment alone 1-h post-treatment (Kephart et al. 2015).

For Days 3–4, 24-h following the previous visit, venipuncture and assessment of flexibility of the right leg and the PPT in the right vastus lateralis was performed as described above. Participants then performed the HIIT described during Day 2 and were treated for 1 h with either EPC or sham.

Days 5–6 (treatments only)

For Days 5–6 (which occurred after the three consecutive HIIT sessions), venous blood sampling and assessment of flexibility of the right leg and the PPT in the right vastus lateralis was performed via methods described above. Thereafter, participants were again treated for 1 h with either EPC or sham, but no HIIT training was performed.

Day 7 (post-testing)

Participants reported 24 h after Day 6 for post-testing (Day 7). Venipuncture and assessment of flexibility of the right leg and the PPT in the right vastus lateralis was performed via methods described above. Next, a final biopsy from the left vastus lateralis was obtained (POST2), and tissue was processed for protein and RNA analyses as described above. The timing of the POST2 biopsy was chosen (1) in alignment with our previous work on EPC and resistance training (Haun et al. 2017) and (2) in an effort to capture the cumulative effects of the experimental conditions, but not the acute effect of the last HIIT session or EPC/sham treatment. Notably, each biopsy was obtained slightly proximal to the previous one as this sampling sequence may prevent variability due to inflammatory signaling that can occur with multiple biopsy sampling (Van Thienen et al. 2014). Finally, a 6-km time-trial run was completed as described above.

Experimental procedures

Serum and plasma analyses

On the days of blood collection (Day1/PRE, Days 3–7), serum and plasma tubes were centrifuged at 3500×*g* for 5 min at room temperature. Aliquots were then placed in 1.7 mL microcentrifuge tubes and stored at − 80 °C until batch-processing. Human enzyme linked immunosorbent assay kits were used to determine serum concentrations of 8-isoprostane (Item # 516351; Cayman Chemical, Ann Arbor, MI, USA) and plasma levels of C-reactive protein (CRP) (Catalog # 11190; Oxis International Inc., Foster City, CA, USA). An activity assay was used to determine serum levels of CK (Catalog # 3460-07; Bioo Scientific, Austin, TX, USA). All kits were performed according to manufacturer's instructions, individual samples were assayed at least in duplicate, and plates were read using a 96-well spectrophotometer (BioTek, Winooski, VT, USA). In our hands, the coefficient of variation for duplicate/triplicate samples was 8.9, 5.1, and 6.4% for 8-isoprostane, CRP, and CK, respectively. Of note, venipuncture was not performed for serum measures at Day2 (POST1) based on marginal responses at this time point previously observed in the laboratory (unpublished data).

RNA expression analyses

Immediately following muscle extraction, samples were homogenized in 500 µL of Ribozol (Ameresco) and stored at − 80 °C for batch processing. During batch processing, total ribonucleic acid (RNA) isolation occurred according to manufacturer's instructions. RNA concentrations were

subsequently assessed using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA) prior to cDNA synthesis for mRNA analyses. cDNA was reverse transcribed from 1000 ng of total RNA for real time PCR analyses using a commercial cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR was performed using SYBR-green-based methods with gene-specific primers designed using an online primer designer tool (Primer-3Plus, Cambridge, MA, USA). Fold-change values within each subject from the PRE biopsy were performed using the Livak method (Schmittgen and Livak 2008), where $-\Delta\Delta CT = (\text{post-treatment gene of interest} - \text{post-treatment geometric mean of GAPDH and Fbl}) - (\text{pre-treatment gene of interest} - \text{pre-treatment geometric mean of GAPDH and Fbl})$. Following the PCR reaction for each gene, melt curve analyses were performed to ensure that one PCR product was amplified per reaction. A list of targeted markers and PCR primers is listed in Table 1.

Western blotting analyses

Immediately following muscle extraction, samples were homogenized using a tight-fitting micropestle in cell lysis buffer described above, insoluble proteins were removed with centrifugation at 500×*g* for 5 min at 4 °C, and supernatants containing muscle tissue homogenate were collected and stored at − 80 °C. After all participants finished the study, muscle tissue homogenates were batch-assayed for total protein content using a bicinchonic acid (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

Cell lysis homogenates were prepared for Western blotting using 4× Laemmli buffer at 1 µg/µL. Thereafter, 20 µL of prepped samples were loaded onto 12% SDS–polyacrylamide gels (BioRad, Hercules, CA, USA) and subjected to electrophoresis (180 V @ 60 min) using 1× SDS–PAGE running buffer (Ameresco). Proteins were then transferred to polyvinylidene difluoride membranes (BioRad), Ponceau stained and imaged to ensure equal protein loading between lanes. Thereafter, membranes were blocked for 1 h at room temperature with 5% nonfat milk powder. Mouse anti-pan IkBα (1:1,000; Cell Signaling, Catalog #4814), rabbit anti-pan p65/NF-κB (1:1000; Cell Signaling; Catalog #8242), rabbit anti-Fbx32 (atrogin-1; 1:500; Abcam, Cambridge, MA, USA; Catalog #ab74023), rabbit anti-MuRF-1 (1:1000; Abcam; Catalog #ab172479), rabbit anti-Ubiquitin (poly-Ub, 1:1000, Cell Signaling; Catalog #3933), rabbit anti-4HNE (1:1000; Abcam; Catalog #ab46545), rabbit anti-catalase (1:1000; GeneTex, Irvine, CA, USA; Catalog #GTX110704), rabbit anti-GPx (1:1000, Abcam; Catalog #GTX116040), and rabbit anti-SOD2 (1:2000, Abcam; Catalog #GTX116093) were incubated with membranes overnight at 4 °C in 5% bovine serum albumin (BSA), and the following day membranes were incubated with respective horseradish peroxidase-conjugated anti-rabbit (1:2000, Cell Signaling; Catalog #7074) or anti-mouse IgG (1:2000, Cell Signaling, Catalog #7076) or at room temperature for 1 h prior to membrane development. Membrane development

Table 1 Primer sequences for real time PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Inflammation		
IL-6	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT
MCP-1	TCCCAAAGAAGCTGTGATCTTCA	CAGATTCTTGGGTGGAGTGA
TNF-α	TCCTTCAGACACCCTCAACC	AGGCCCCAGTTTGAATTCTT
Metabolism		
PPARGC1A	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC
Proteolysis		
Atrogin-1	ATGTGCGTGTATCGGATGG	AAGGCAGGTCAGTGAAGC
MuRF-1	GCCTTCTTCGCCTTCTCC	AGCTCATACAGACTCAGTTCC
Redox status		
Catalase	CTGACTACGGGAGCCACATC	AGATCCGGACTGCACAAAGG
GPx	ACGAGGGAGGAACACCTGAT	TCTGGCAGAGACTGGGATCA
SOD2	GTTGGGGTTGGCTTGGTTTC	GCCTGTTGTTCCTTGCAGTG
Housekeeping genes		
Fbl	CCCACACCTTCCTGCGTAAT	GCTGAGGCTGTGGAGTCAAT
GAPDH	AACCTGCCAAATATGATGAC	TCATACCAGGAAATGAGCTT

All primers were designed using PrimerPlus3 (Cambridge, MA, USA) and BLASTed against other potential mRNA targets using the online NCBI Nucleotide database (Bethesda, MD). IL-6, interleukin-6, MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor α; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; MuRF-1, muscle RING finger 1; GPx, glutathione peroxidase, SOD2, superoxide dismutase 2; Fbl, fibrillarin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

was performed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; Millipore, Billerica, MA, USA), and band densitometry was performed through the use of a gel documentation system and associated densitometry software (UVP, Upland, CA, USA). Of note, the densitometry values for all protein targets were normalized to Ponceau densities.

Immunoprecipitation for determination of acetylated PGC-1α

To measure acetylated (Ac) PGC-1α levels, 200 µL of cell lysate was incubated with rabbit anti-acetylated lysine antibody (1:1000, Cell Signaling; Catalog #9441) overnight at 4 °C. The following day, protein A agarose beads (20 µL of 50% bead slurry, Cell Signaling) were added to antibody–lysate mixtures and rocked 3 h at 4 °C. Samples were then microcentrifuged for 30 s at 4 °C, supernatants were discarded, and the pellet was washed with 500 µL of cell lysis buffer (recipe described above, Cell Signaling). After two additional repeat washes, samples were microcentrifuged for 30 s at 4 °C, supernatants were discarded, beads were resuspended with 20 µL of 4× Laemmli buffer, and samples were heated at 100 °C for 5 min. Thereafter, samples were loaded onto 12% SDS–polyacrylamide gels (BioRad, Hercules, CA, USA) and subjected to electrophoresis and transfer as described above. Membranes were incubated with rabbit anti-PGC-1α (1:1000; Abcam, Catalog #ab54481) overnight, horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling, Catalog #7074) at room temperature for 1 h prior to membrane development, and developed as described above. The densitometry values for Ac-PGC-1α were normalized to Ponceau densities, and these values were normalized to PRE values to obtain fold-change values from 1.00. Ac-PGC-1α raw density values were also normalized to raw PGC-1α values to obtain Ac-PGC-1α/PGC-1α ratios.

Statistics

For all statistical analyses, an alpha level of $p \leq 0.05$ was required for statistical significance. Independent *t* tests were performed for between-groups (EPC vs. sham) comparisons of age, height, weight, BMI, and VO_{2peak} at study entry. Respective to the primary aims of the present study, (1) to evaluate change in muscle soreness, flexibility and serum measures for all sampled time points with EPC compared to sham 2 × 6 [group (EPC, sham) × time (PRE and Days3–7)] repeated measures ANOVAs were performed using absolute values; (2) to compare the acute effects of a single bout of HIIT followed by EPC and sham on change in skeletal muscle markers, between groups Welch–Satterthwaite independent *t* tests on change from PRE at POST1 were performed, and (3) to compare the change in skeletal muscle markers and 6-km run performance 24 h after three consecutive days of HIIT and three additional days of recovery with daily EPC treatment compared to daily sham treatment, Welch–Satterthwaite independent *t* tests were performed on change from PRE at POST2 were performed. For ANOVAs, Mauchly's test of sphericity was performed to assure equal variances and normally distributed data. In the event that sphericity was not met, Huynh–Feldt correction was applied to hypothesis testing. When a significant main effect of time (within-subjects factor) was observed, each time point was compared to PRE using Student's paired *t* tests. In these instances, Bonferroni adjustments were applied ($\alpha/5 = 0.01$). Grouped data are presented as mean ± standard error and data respective to effect sizes/post-hoc comparisons are presented as the mean difference and [95% confidence interval (lower limit, upper limit)]. All statistics were performed using SPSS v22.0 (Chicago, IL, USA).

Results

Participant characteristics

Participant characteristics are presented in Table 2. No significant between-group differences were observed for age, height, body mass, body mass index, or VO_{2peak} ($p > 0.05$).

Functional outcomes

Data regarding effects of EPC and sham on functional outcomes are presented in Fig. 2. For muscle soreness (i.e., PPT) a significant main effect of time, but no main effect of group or time*group interaction, was observed at all sites as well as for the 3-site average (Fig. 2a–d). Across both treatment groups, the PPT was significantly reduced relative to PRE at Day 3 [− 16.5 N (− 8.8, − 24.2); $p < 0.001$] and Day 5 [− 13.5 N (− 5.4, − 21.6); $p = 0.003$] for the 3-site average. No main effects or interaction were observed for changes in flexibility (i.e., modified kneeling lunge knee angle; Fig. 2e). Finally, change from PRE at POST2 in 6 km time trial performance was not significantly different between groups (Fig. 2f).

Blood markers of muscle damage, oxidative stress and inflammation

Serum CK activity, a marker of muscle damage, demonstrated a main effect of time but no main effect of group or group × time interaction (Fig. 3a). Across both groups, serum CK was significantly higher relative to PRE at Day 3 [+ 38.7 U/L (+ 22.5, + 54.9); $p < 0.001$], Day 4 [+ 57.2 U/L (+ 16.8, + 97.5); $p = 0.009$], and Day 5 [+ 32.1 U/L (+ 14.6,

Table 2 Participant characteristics

	Overall ($n=18$)	EPC ($n=9$)	Sham ($n=9$)	p value (EPC vs. sham)
Age (years)	21.1 ± 0.4	21.0 ± 0.4	21.1 ± 0.6	0.872
Height (m)	1.79 ± 0.02	1.79 ± 0.03	1.79 ± 0.03	0.952
Body mass (kg)	82.4 ± 1.6	80.1 ± 2.2	85.0 ± 2.2	0.130
BMI (kg/m^2)	25.7 ± 0.5	24.9 ± 0.7	26.5 ± 0.5	0.093
VO_{2peak} (mL/kg/min)	51.3 ± 0.9	51.5 ± 1.5	51.0 ± 0.9	0.785

Values are mean ± sem. p values from between-groups intendent t tests

BMI body mass index; VO_2peak peak 30-s average VO_2 observed during graded exercise testing

+49.6); $p=0.001$]. For 8-isoprostane (Fig. 3b), a marker of lipid peroxidation, and hsCRP (Fig. 3c), a marker of inflammation, no significant main effects or time × group interactions were observed.

Skeletal muscle gene expression

Targeted gene expression responses to the protocol herein are presented in Table 3. Change in atrogin-1 gene expression from PRE were significantly different between groups at the POST1 time point [mean difference: −0.28 (−0.19, −0.37); $p=0.018$], but not the POST2 time point ($p=0.313$). Atrogin-1 mRNA levels decreased 19.7 ± 8.1% from PRE at the POST1 time point in the EPC group and increased 7.7 ± 5.9% in the sham group. No other genes were found to be significantly different between groups at either the POST1 or POST2 time point.

Skeletal muscle protein expression patterns

Targeted protein expression responses to the protocol herein are presented in Table 4. No significant between-group differences for change from PRE were observed at the POST1 or POST2 time point for inflammation, metabolism, or redox status related proteins. Change from PRE in atrogin-1 protein expression was significantly different between groups at the POST2 time point [mean difference: 128% (36%, 217%); $p=0.013$]. Atrogin-1 decreased 31.8 ± 7.5% from PRE at the POST2 time point in the EPC group and increased 96.0 ± 34.7% in the sham group (Fig. 4a). No significant between groups differences for change from PRE were observed at either the POST1 or POST2 time point for MuRF-1 protein expression (Fig. 4b). Change from PRE in poly-Ub proteins was significantly different between groups at the POST1 [mean difference: 60.7% (1.3%, 120.2%); $p=0.046$] and POST2 time points [mean difference: 55.1% (4.2%, 106.0%); $p=0.037$]. Change from PRE in Poly-Ub proteins was −26 ± 10.3 and −33.7 ± 17.2% at the POST1 and POST2, respectively, in the EPC group and +34.8 ± 28.5 and +21.4 ± 14.9% at POST1 and POST 2, respectively, in the sham group (Fig. 4c).

Discussion

We consider the following as primary findings of this investigation: (1) no significant differences existed between groups in functional variables related to exercise performance (e.g., flexibility, soreness, 6-km run time) or serum measures of muscle damage, oxidative stress and inflammation, (2) compared to sham, 1-h of EPC treatment following a single bout of HIIT was associated with lower atrogin-1 gene expression and poly-Ub protein concentrations, and (3) compared to daily sham treatment, daily EPC treatment was associated with significantly less atrogin-1 and poly-Ub protein expression 24 h after three consecutive days of HIIT and three additional days of recovery.

Functional measures

Flexibility, muscle soreness, and 6-km run time trial performance changes were not significantly different between groups. The lack of an effect on flexibility and muscle soreness, compared to the differences observed in our resistance exercise study involving EPC, is likely associated with the greater eccentric component and tissue-damaging forces involved in heavy resistance exercise (Haun et al. 2017). Indeed, Nosaka et al. reported significantly worsened maximal isometric force production and resting joint angles along with significantly greater muscle soreness and plasma CK activity when subjects completed 12 maximum eccentric muscle actions of elbow flexion every 15 s for 3 min compared to a group completing 2 h of consistent elbow flexion with a load corresponding to ~10% of maximal isometric force (Nosaka et al. 2002). Thus, the nature of the exercise, and resultant magnitude of change from baseline in flexibility and muscle soreness in this study, likely explains the lack of differences in these parameters and seems to have prohibited identification of a treatment effect of EPC.

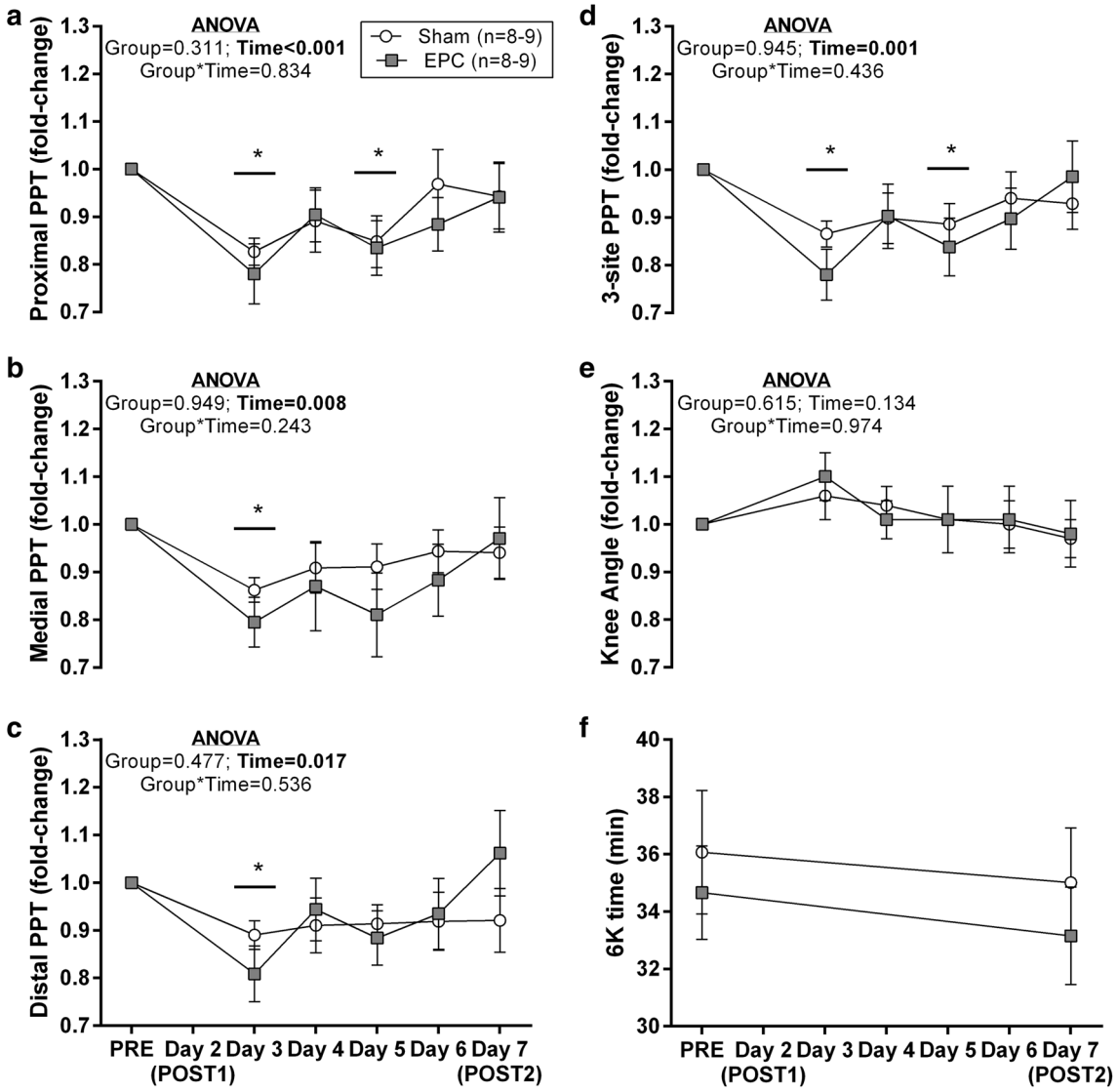

Fig. 2 Functional changes in response to EPC and sham throughout the protocol utilized herein. Muscle soreness [i.e., pressure-to-pain threshold (PPT)] along the right vastus lateralis at a proximal, medial, and distal site is presented in **a**–**c** and a composite 3-site average is presented in **d**. Flexibility assessed via right knee range of motion in a modified lunge position is presented in **e**. 6-km run time trial performance is presented in **f**. For all panels data are presented as mean fold-change from PRE ± standard error. Repeated measures ANOVAs for PPT and flexibility measures and between groups independent *t* tests for change from PRE in 6 km run time at the POST2 time point were performed with an $\alpha \leq 0.05$ required for statistical significance. Main effects of time are presented as bold *p* values. Post-hoc testing for a main effect of time was performed using Student's paired *t* tests and an $\alpha \leq 0.01$ was required for statistical significance. *, time point(s) (collapsed across groups) significantly different from PRE

In addition, on average, 6-km run times were marginally reduced at POST2 ($-3.5 \pm 1.1\%$) indicating that the protocol "missed the mark" in terms of evaluating performance at a time point when it was substantially depressed post-high intensity training.

Serum measures

In this investigation, serum CK approximated the magnitude of muscle damage secondary to disruption of myocyte structure at the sarcolemma and Z-disk (Brancaccio et al. 2007), serum hsCRP was used as a surrogate of systemic inflammation given its acute synthesis in defensive or adaptive responses to inflammatory stimuli (Black et al. 2004), and 8-isoprostane levels served as a proxy of the magnitude of systemic oxidative stress (Nikolaidis et al. 2011). A significant main effect of time for serum CK levels was observed which served as confirmation that the HIIT and protocol utilized herein elicited significant myocellular damage. However, similar to our findings with resistance training

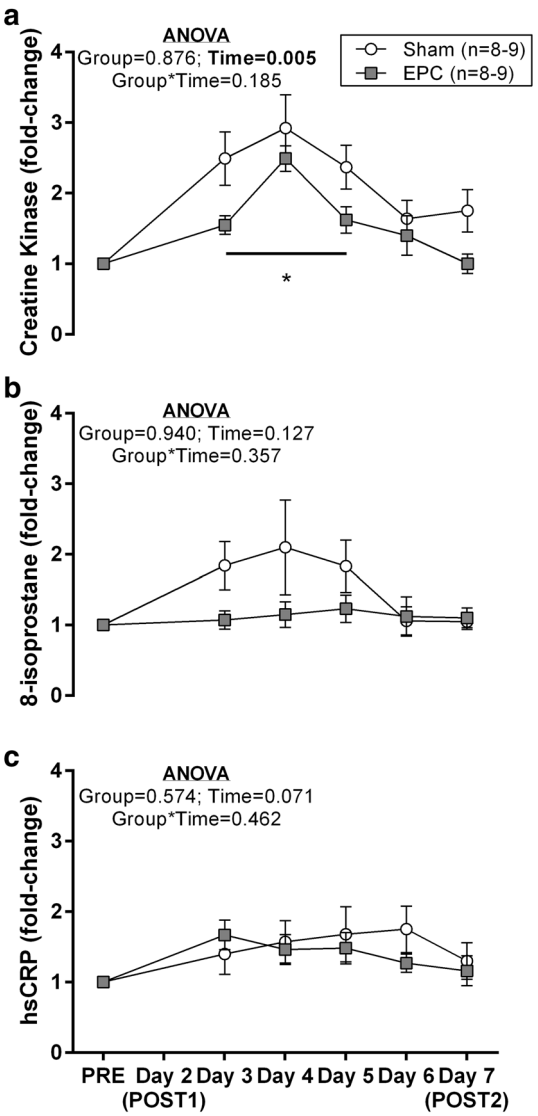

Fig. 3 Humoral markers of muscle damage, oxidative stress, and inflammation in response to EPC and sham throughout the protocol utilized herein. Creatine kinase (CK; **a**), 8-isoprostane (**b**), and high sensitivity C-reactive protein (hsCRP; **c**) concentrations in the blood were measured at baseline (PRE/Day1) and on Days 3–7 of the protocol. Data are presented as mean fold-change from PRE ± standard error. Repeated measures ANOVAs were performed with an $\alpha \leq 0.05$ required for statistical significance. Main effects of time are presented as bold *p* values. Post-hoc testing for a main effect of time was performed using Student's paired *t* tests and an $\alpha \leq 0.01$ was required for statistical significance. *, time point(s) (collapsed across groups) significantly different from PRE

and EPC, there was no statistically significant effect of EPC treatment on hsCRP and CK responses associated with the protocol. In addition, no statistically significant group × time interaction was observed for serum 8-isoprostane concentrations. Given our previous observations of significant reductions in local skeletal muscle markers of oxidative stress (i.e., 4HNE) when EPC is used with resistance training (Haun et al. 2017), this was surprising. However, in both the sham and EPC groups there was no increase in local (i.e., skeletal muscle) 4HNE expression which is in contrast with our previous findings with resistance training (Haun et al. 2017). Thus, the relatively limited level of induced myocellular oxidative stress we observed herein likely contributes to these observed findings.

mRNA and protein expression

Interestingly, atrogin-1 mRNA expression was the only significantly different mRNA expression pattern observed between groups with relatively lower levels at the POST1 time point in the EPC group compared to sham (Table 3). Atrogin-1 is an E3 ligase involved in tagging proteins for degradation in the 20S proteasome core of the ubiquitin/proteasome pathway (Louis et al. 2007). Although E2 and E3 ligase gene expression does not appear to correlate with their activity (Lecker et al. 1999), in this study, the relatively lower atrogin-1 gene expression with EPC treatment at the POST1 time point was associated with relatively lower atrogin-1 protein expression at the POST2 time-point compared to sham (Fig. 4a). Moreover, change from PRE in poly-Ub proteins at the POST1 and POST2 time points was significantly different between-groups with an increase being observed in the sham group compared to a reduction in the EPC group suggesting a relatively less proteolytic-related signaling and events. It is thought that the response to intense endurance exercise of sufficient duration initially involves a decrease in muscle protein synthesis and an increase in proteolysis (Rennie and Tipton 2000; Kee et al. 2002). Although the exact mechanism is unclear in this investigation, we posit that suppression of proteolytic markers with EPC compared to sham is due to potentiation of hormone, nutrient and metabolite delivery and clearance to/from the skeletal muscle tissue mediated by the repeated compression relaxation cycles occurring along the lower limbs during EPC treatment. Indeed, perturbations in lymphatic clearance (Vincent et al. 2005), endothelial surface area for diffusion via arteriolar vasodilation and/or microcirculatory blood flow (Kolka and Bergman 2012), and alterations in interstitial pressures (Reed and Rubin 2010) in response to EPC treatment may augment hormone and nutrient delivery/uptake in the skeletal muscle that can down-regulate ubiquitin–proteasome catalyzed skeletal muscle proteolysis (Biolo et al. 1997, 1999; Chotechuang et al. 2011; Tesseraud et al. 2007). We have previously noted that skeletal muscle blood flow (Martin et al. 2016a) and vascular reactivity (Martin et al. 2015a) are increased with EPC. Moreover, we have also observed significantly reduced poly-Ub proteins with EPC compared to sham with voluminous resistance training (Haun et al. 2017). Granted, we did not investigate the effects of EPC on muscle protein

Table 3 Effects of EPC following high intensity interval training on skeletal muscle gene expression

Marker (s)	Sham-POST1	Sham-POST2	EPC-POST1	EPC-POST2	*p* values (EPC vs. sham)	
					POST1	POST2
Inflammation						
IL-6	1.38 ± 0.31	1.59 ± 0.30	1.56 ± 0.33	1.13 ± 0.27	0.698	0.270
MCP-1	1.67 ± 0.36	1.10 ± 0.21	1.74 ± 0.77	1.78 ± 0.45	0.937	0.202
TNF-α	1.06 ± 0.19	1.65 ± 0.36	1.05 ± 0.25	1.17 ± 0.35	0.975	0.369
Metabolism						
PPARGC1A	2.20 ± 0.40	1.16 ± 0.09	2.48 ± 0.35	0.94 ± 0.11	0.611	0.151
Proteolysis						
Atrogin-1	1.08 ± 0.06	1.05 ± 0.10	0.80 ± 0.08	0.90 ± 0.11	**0.018**	0.313
MuRF-1	1.21 ± 0.24	0.95 ± 0.16	1.11 ± 0.20	1.05 ± 0.21	0.770	0.758
Redox status						
Catalase	1.19 ± 0.09	1.08 ± 0.08	1.09 ± 0.14	1.00 ± 0.16	0.579	0.653
GPx	1.08 ± 0.10	1.01 ± 0.10	1.11 ± 0.03	1.10 ± 0.09	0.783	0.495
SOD2	0.88 ± 0.13	0.97 ± 0.13	1.22 ± 0.15	1.11 ± 0.20	0.108	0.549

All data are expressed as fold change from 1.00 (mean ± standard error, n = 7–9 subjects per target). Statistical comparisons from between-groups independent t tests (significance indicated in bold). Other notes: Gene abbreviations are in Table 1 which denotes primer sequences employed herein

Table 4 Effects of EPC following high intensity interval training on skeletal muscle protein expression

Marker (s)	Sham-POST1	Sham-POST2	EPC-POST1	EPC-POST2	*p* values (EPC vs. sham)	
					POST1	POST2
Inflammation						
pan-IκBα	0.93 ± 0.11	0.83 ± 0.10	0.85 ± 0.15	1.03 ± 0.10	0.702	0.568
pan-NF-κB p65	1.01 ± 0.11	0.74 ± 0.10	0.85 ± 0.18	0.97 ± 0.15	0.452	0.233
Metabolism						
PGC-1α	1.36 ± 0.15	1.15 ± 0.07	1.16 ± 0.25	1.17 ± 0.36	0.531	0.956
Ac-PGC-1α	2.02 ± 0.64	1.41 ± 0.44	1.75 ± 0.51	1.24 ± 0.44	0.753	0.792
Ac-PGC-1α/ PGC-1α	1.17 ± 0.47	1.12 ± 0.41	0.99 ± 0.32	1.28 ± 0.41	0.760	0.784
Proteolysis						
Atrogin-1	1.18 ± 0.36	1.96 ± 0.35	0.83 ± 0.16	0.68 ± 0.08	0.372	**0.013**
MuRF-1	1.35 ± 0.28	1.60 ± 0.46	1.29 ± 0.15	1.16 ± 0.14	0.854	0.398
Poly-Ub	1.35 ± 0.29	1.21 ± 0.15	0.74 ± 0.10	0.66 ± 0.17	**0.046**	**0.037**
Redox status						
4HNE	0.97 ± 0.08	0.85 ± 0.08	1.09 ± 0.08	0.93 ± 0.06	0.287	0.469
Catalase	0.84 ± 0.13	1.02 ± 0.10	1.09 ± 0.07	1.16 ± 0.08	0.126	0.326
GPx	1.00 ± 0.29	0.90 ± 0.28	0.92 ± 0.29	0.93 ± 0.40	0.848	0.942
SOD2	1.04 ± 0.15	1.07 ± 0.08	1.00 ± 0.10	0.96 ± 0.11	0.853	0.446

All data are expressed as fold change from 1.00 (mean ± standard error, n = 7–9 subjects per target). Statistical comparisons from between-groups independent t tests (significance indicated in bold)

IκBα nuclear factor of kappa B (NF-κB) inhibitor alpha, *PGC-1α* peroxisome proliferator-activated receptor coactivator 1-alpha, *Ac-* acetylated, *MuRF-1* muscle RING finger-1, *poly-Ub* poly-ubiquitinated protein, *4HNE* 4-hydroxynonenal, *GPx* glutathione peroxidase, *SOD2* superoxide dismutase 2

synthesis rates herein and cannot conclude if the reduction in markers of proteolysis results in improved skeletal muscle protein balance. However, the results suggest that EPC could positively impact recovery-adaptation through reductions in muscle catabolism in response to training.

Limitations

The present study is not without limitations. First, vastus lateralis skeletal muscle biopsies were only collected at the PRE, POST1 and POST2 time points. Thus, the cumulative

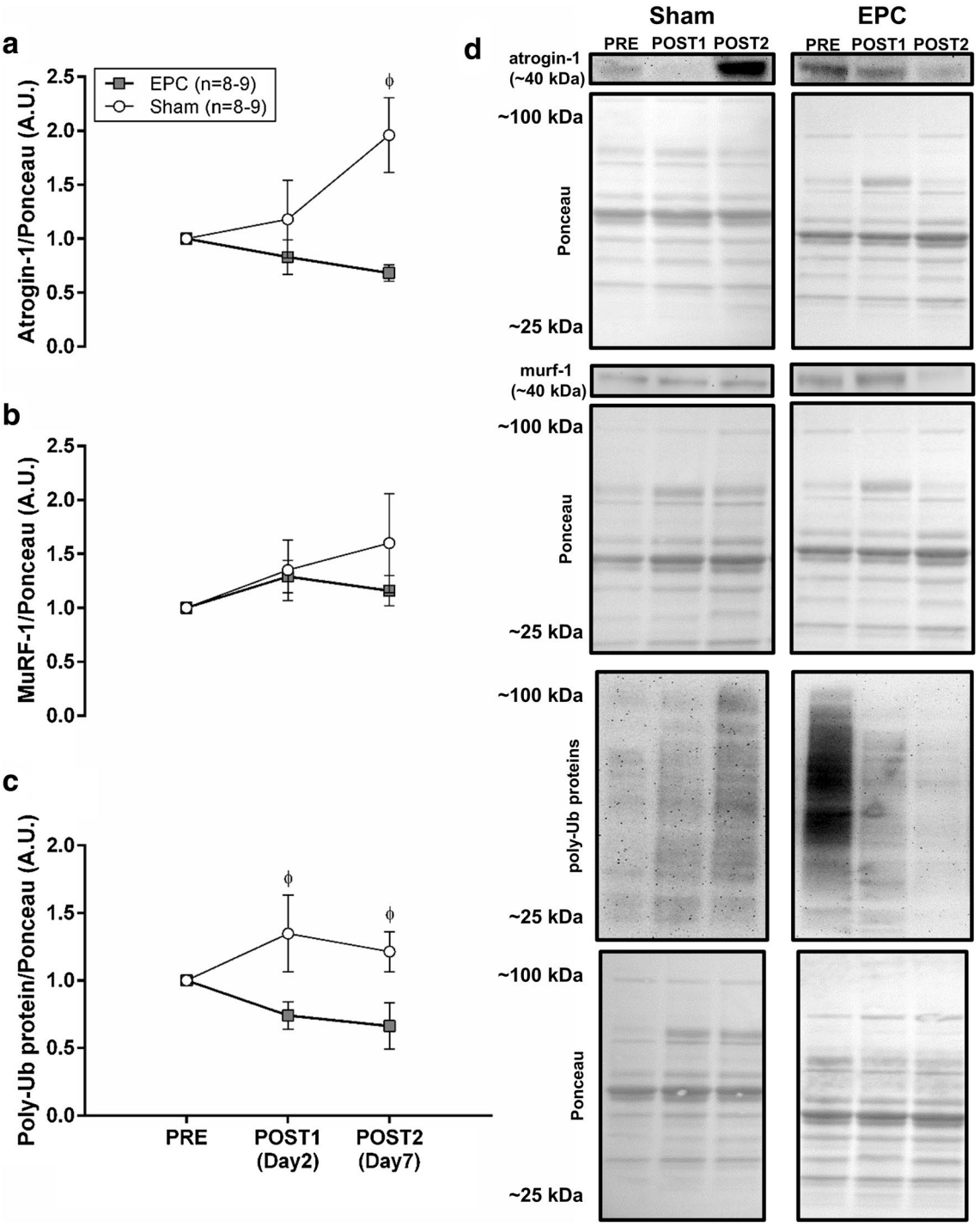

Fig. 4 Protein expression patterns related to proteolytic signaling. At baseline (PRE), 1 h following high intensity interval training (HIIT) and treatment with EPC or sham (POST1), and 24 h following three consecutive days of HIIT and treatment with EPC or sham and two additional, consecutive days of treatment with EPC or sham (POST2) protein expression patterns related to proteolytic signaling were probed. Western blot analysis of protein concentrations in vastus lateralis biopsy samples are presented in **a** atrogin-1, **b** MuRF-1, and **c** poly-ubiqutinated (poly-Ub) proteins. Representative images and respective Ponceau images for all proteins are presented in **d**. Values represent the mean fold change of protein expression normalized to ponceau from PRE ± standard error. Change from PRE at POST1 and POST2 were compared using independent *t* tests. ϕ, significantly different between groups ($p < 0.05$)

effects of three consecutive days of HIIT training with and without EPC treatment on skeletal muscle gene expression and protein concentrations remain unknown. Notably, we chose to evaluate the acute effects of HIIT and EPC or sham after the first session given that the physiological response to repeated exercise on successive days is likely less robust (McHugh 2003). Second, with respect to the acute effects of HIIT and EPC or sham, biopsies were only taken at 1-h post EPC/sham treatment (2-h post HIIT). Thus, in consideration of the timing and transient nature of cellular signaling and gene expression responses, it is likely that we did not capture all of the signals associated with the observed physiological response(s). Third, biopsies corresponding to the PRE time point commenced 1 week prior to the POST1 time point. Therefore, it is possible that some variability in gene expression and protein concentration existed due to the time between the PRE and POST1 measures. However, we elected to use the 1-week of time between PRE and POST1 to (1) allow complete recovery from the initial 6-km run and (2) limit biochemical artifact secondary to myocellular disturbances from the biopsy procedure itself (Van Thienen et al. 2014). Finally, we did not conduct any formal dietary analysis. Indeed, although participants were instructed to maintain normal dietary habits throughout the study, without an objective measure of nutritional intake during the study the potential influence of dietary behaviors cannot be excluded.

Conclusion(s)

In conclusion, compared to sham, dynamic EPC treatment of the lower limbs concurrently with HIIT and the protocol herein reduces markers of proteolysis in skeletal muscle tissue, but not flexibility, soreness, or 6-km time trial performance. More research clarifying the specific mechanisms whereby EPC reduces markers of oxidative stress and proteolysis is warranted. Additionally, at present, investigations into the long-term use of EPC in the context of ongoing training for athletic performance is non-existent. Future studies can provide insight into the long-term effects of EPC and allow a better understanding of specific dose–response relationships of EPC and adaptations influencing exercise and/or sport performance.

Acknowledgements Partial reagent and participant compensation costs (50%) were paid through a contract awarded to J.S.M. by Normatec (Newton Centre, MA, USA). The funders did have a role in study design, but had no role in data collection and analysis, decision to publish, or preparation of the manuscript. The results of this study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The authors wish to thank the participants for their time and compliance with demands associated with the study protocol.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

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