A single 60-min bout of peristaltic pulse external pneumatic compression transiently upregulates phosphorylated ribosomal protein s6

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Accepted for publication
Received 1 July 2015;
accepted 9 December 2015

Key words
Akt-mTOR signalling; external pneumatic compression; phosphorylation; rps6; skeletal muscle

Summary

We investigated whether a single 60-min bout of whole leg, peristaltic pulse external pneumatic compression (EPC) altered select growth factor-related mRNAs and/or various phospho(p)-proteins related to cell growth, proliferation, inflammation and apoptosis signalling (e.g. Akt-mTOR, Jak-Stat). Ten participants (8 males, 2 females; aged 22·2 ± 0·4 years) reported to the laboratory 4 h postprandial, and *vastus lateralis* muscle biopsies were obtained prior to (PRE), 1 h and 4 h post-EPC treatment. mRNA expression was analysed using real-time RT-PCR and phosphophorylated and cleaved proteins were analysed using an antibody array. No changes in selected growth factor-related mRNAs were observed following EPC. All p-proteins significantly altered by EPC decreased, except for p-rps6 (Ser235/236) which increased 31% 1 h post-EPC compared to PRE levels ($P = 0·016$). Notable decreases also included p-BAD (Ser112; −28%, $P = 0·004$) at 4 h post-EPC compared to PRE levels. In summary, an acute bout of EPC transiently upregulates p-rps6 as well as affecting other markers in the Akt-mTOR signalling cascade. Future research should characterize whether chronic EPC application promotes alterations in lower-limb musculature and/or enhances exercise-induced training adaptations.

Introduction

Lower-limb compression via a specialized garment whereby intramuscular pressures are elevated and fluid movement gradients are acutely altered has been applied in a variety of clinical settings such as lymphedema, venous insufficiency and venous ulcers (O'Donnell *et al.*, 1979; Zelikovski *et al.*, 1993; Barnett, 2006). However, the use of compressive therapies beyond clinical applications continues to gain traction as it has been posited as a viable recovery method in the athletic sphere.

Lower-limb, static compression garments have been shown to lower creatine kinase (CK), a marker of muscle damage, 1·5 and 3·5 days following rugby participation compared to a passive recovery method (Gill *et al.*, 2006). Likewise, static compression garments have been shown to lower posteccentric exercise CK levels, preserve range of motion in the elbow flexors, reduce perceived soreness and swelling and promote force production recovery (Kraemer *et al.*, 2001). However, other investigations have refuted the application of static compression garments on improving sports performance, postexercise blood lactate clearance and oxygen consumption, and/or postexercise markers of muscle damage (CK) and/or recovery (glycogen resynthesis) (Berry *et al.*, 1990; Duffield *et al.*, 2010; Keck *et al.*, 2015).

Lower pressure (≤120 mmHg) dynamic compression therapies (e.g. peristaltic pulse external pneumatic compression [EPC]) have also gained in popularity amongst athletes relative to sports performance and recovery. Specific to dynamic compression therapies, a single 15-min EPC treatment following resistance training in elite athletes has been shown to immediately, and persistently, reduce the pressure-to-pain threshold in the lower limbs (Sands *et al.*, 2015). Moreover, 30 min of EPC treatment during recovery from supramaximal exercise bouts has been shown to improve blood lactate clearance compared to passive recovery (Martin *et al.*, 2015b). However, there is limited evidence in the literature exploring how low pressure EPC devices mechanistically affect cellular signalling in skeletal muscle. Recently, we demonstrated that a single 60-min bout of EPC acutely increases skeletal muscle gene expression pertinent to cellular energy metabolism (i.e. PGC-1α mRNA) (Kephart *et al.*, 2015), though the effects of EPC on cellular signalling associated with skeletal muscle growth and/or tissue repair that may be applicable to recovery models

in the athletic paradigm have not yet been characterized. Thus, the purpose of this study was to examine whether an acute bout of EPC affects selected growth factor-related mRNA markers as well as phosphophorylated and/or cleaved proteins associated with cellular growth [protein kinase B (Akt)-mechanistic target of rapamycin (mTOR], cell proliferation [mitogen-activated protein kinase (MAPK)], inflammation [Jak-Stat] and apoptosis signalling from 1 to 4 h following a single 60-min bout of EPC. While exploratory in nature, we hypothesized that a single EPC treatment would improve markers of cellular growth, cell proliferation, inflammation and/or cellular stress. A chief outcome of this study was to further characterize the acute effects of EPC on gene expression and cellular signalling and guide future EPC investigations.

Methods

Ethical approval

All procedures described herein were approved by Auburn University's Institutional Review Board and conformed to the standards set by the latest revision of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their voluntary participation in the study. All participant results have been anonymized, and participant identities cannot be recognized in this manuscript in any way. Finally, all laboratory health and safety procedures were complied with in the course of the experimental work reported herein.

Participants

Ten volunteers were enrolled in this study ($n = 8$ males, $n = 2$ females). Eligibility criteria included the following: (i) participants were apparently healthy, medication-free and had no history of blood clotting issues; and (ii) participants had not consumed ergogenic nutritional supplements for at least 1 month prior to the compression bout.

Subject characteristics were as follows (mean ± SD): age, 22·0 ± 0·4 years; height, 178 ± 3 cm; body mass, 77·3 ± 3·1 kg; and BMI, 24·3 ± 0·8 kg m^{-2}. Subjects were recreationally active and self-reported activity levels, on average, of 90 ± 36 min $week^{-1}$ and 149 ± 48 min $week^{-1}$ of moderate intensity aerobic and resistance training exercise, respectively.

Compression protocol and skeletal muscle biopsies

On the day of testing, participants were instructed to report to the laboratory following 4 h devoid of food and/or caffeine-containing beverages and at least 24 h being refrained from rigorous physical activity and alcohol and tobacco use. Participants were then instructed to lay in a supine position on a treatment table whereby a pre-EPC (PRE) percutaneous skeletal muscle biopsy was obtained from the left *vastus lateralis* midway between the patella and iliac crest using a 5-gauge needle with suction and sterile laboratory procedures. Briefly, 1·5 ml of 1% lidocaine was injected subcutaneously above the skeletal muscle fascia prior to making a small pilot incision for needle intrusion. The biopsy needle was then inserted at a depth just beyond the fascia, and approximately 100–150 mg of skeletal muscle was removed using a double-chop method and applied suction. Extracted tissue was immediately blotted of visible blood using sterile gauze pads and had all visible fat and connective tissue removed. Thereafter, approximately 50 mg tissue was immediately placed in a 1·7-ml polypropylene tube containing 500 µl of cell lysis buffer (Cell Signaling, Danvers, MA, USA) spiked with 1× phosphatase II and III inhibitors (G Biosciences, Saint Louis, MO, USA) and processed for protein analyses as described below, 10–20 mg of muscle was placed in a 1·7-ml polypropylene tube containing 500 µl of Ribozol (Ameresco, OH, USA) for mRNA analyses as described below, and remaining tissue was snap-frozen in liquid nitrogen and subsequently stored at −80°C.

After the initial skeletal muscle biopsy, EPC (NormaTec, Newton, MA, USA) was applied only to the right leg for 60 min. The EPC device 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 preprogrammed 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 that we have previously investigated and described (Kephart *et al.*, 2015; Martin *et al.*, 2015a, b). In brief, beginning with the most distal chamber, inflation occurs and the chamber 'pulses' (slight variations in pressure) for 30 s after which pressure is held constant (~70 mmHg) to prevent backflow. The same process then occurs in each of the next highest zones individually. Notably, a maximum of only two distal chambers are held at constant pressure to facilitate greater rest time (no compression) in each chamber. After the most proximal zone completes its cycle, all zones are completely deflated for 30 s. This entire cycle of compression is repeated continuously over the course of a single 60-min treatment session (for a total of 20 complete cycles).

After the 60-min EPC protocol, the legging was removed and subjects passively rested on a treatment table. At 1 and 4 h following the completion of the 60-min EPC treatment (i.e. post-EPC), two separate skeletal muscle biopsies, spaced 5 cm apart, were obtained from the right *vastus lateralis* to reduce the potential inflammatory effects that could have carried over from the 1 h post-EPC biopsy. Moreover, the 1 h post-EPC biopsy was obtained more distal and the 4 h post-EPC biopsy more proximal due to prior literature showing this sampling sequence prevents inflammatory signalling events

that may occur with a two-site biopsy approach (Van Thienen *et al.*, 2014). Of note, the pre-EPC biopsy was obtained from the left leg and the post-EPC biopsies were obtained from the right leg in order to ensure that EPC did not forcefully open the pre-EPC biopsy site. It should also be noted that participants remained in a supine position during and after the compression bout to control for physical activity levels during the trial.

RNA isolation and mRNA analyses

About 10–20 mg portions of muscle biopsy samples were homogenized in 500 μl of Ribozol (Ameresco) and stored at −80°C for batch processing. During batch processing, total 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. About 500 ng of total RNA was reverse transcribed into cDNA 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 (Primer3Plus, Cambridge, MA, USA) or from previously published primer sequences (denoted in Table 1). The following mRNAs were analysed: nerve growth factor (NGF), mechano growth factor (MGF), neurotrophin-3 (NTF-3) and myostatin (MSTN). The geometric mean of beta2-microglobulin (B2M) and 28S rRNA (28S) was used as a normalizer given that this provided stability of the data throughout all time points.

Fold-change values from the pre-EPC baseline biopsy were performed using the Livak method (i.e. $2^{-\Delta\Delta CT}$ assuming 100% primer binding efficiency) (Livak & Schmittgen, 2001), where – ΔΔCT = (post-EPC gene of interest – post-EPC geometric mean of B2M and 28S) − (pre-EPC gene of interest – pre-EPC geometric mean of B2M and 28S). Following the PCR for each gene, melting curve analyses were performed to ensure that one PCR product was amplified per reaction. Primer sequences are presented in Table 1.

Protein isolation and directed phospho-proteomics

Immediately following muscle extraction, samples were homogenized using a tight-fitting micropestle, 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 BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and were diluted to 1·0 μg μl^{-1} using additional cell lysis buffer (Cell Signalling).

PathScan® Intracellular Signaling Antibody Array Kits (Chemiluminescent Readout; Cell Signaling, Danvers, MA) containing glass slides spotted with antibodies were utilized to detect phosphorylated proteins within the muscle tissue homogenates of each sample belonging to the Akt-mTOR, MAPK, Jak-Stat and cellular stress signalling pathways. Assay targets included phospho(p)-extracellular signal-related kinases [ERK1/2] (Thr202/Tyr204), p-Stat1 (Tyr701), p-Stat3 (Tyr705), p-Akt (Thr308), p-Akt (Ser473), p-AMP-activated protein kinase [AMPK]α (Thr172), phosphorylated ribosomal protein S6 [p-rps6 (Ser235/236)], p-mTOR (Ser2448), p-heat-shock protein [HSP]27 (Ser78), p-Bad (Ser112), p-p70s6k (Thr389), p-proline-rich Akt substrate of 40 kDa [PRAS40] (Thr246), p-p53 (Ser15), p-p38 MAPK (Thr180/Tyr182), p-Jun N-terminal kinase [JNK] (Thr183/Tyr185) and p-glycogen synthase kinase-3 beta [GSK-3β] (Ser9). Cleaved poly ADP-ribose polymerase [PARP] (Asp214) and cleaved Caspase 3 (Asp175) were also represented on the array. These phospho-signalling targets are readouts for insulin signalling [p-Akt (Thr308) and p-Akt (Ser473)], skeletal muscle protein synthesis [p-mTOR (Ser2448), p-p70s6k (Thr389)

Table 1 Effects of EPC on select growth factor-related mRNA expression patterns and primer sequences for RT-PCR.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
NGF	GAAAATTTGCAGCTGCCTTC	ACTCCACTGCCATTCTCCAC
MGF	CGAAGTCTCAGAGAAGGAAAGG	ACAGGTAACTCGTGCAGAGC
NTF-3	CACAGACTCAGCTGCCAGAG	TGAGGGAATTGAGCGAGTCT
MSTN	GACCAGGAGAAGATGGGCTGAATCGTT	CTCATCACAGTCAAGACCAAAATCCCTT
B2M (HKG1)	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG
28S (HKG2)	ACCTGTCTCACGACGGTCTA	GCGTTGGATTGTTCACCCAC

Gene	PRE	1 h post-EPC	4 h post-EPC
NGF	1·00 ± 0·16	1·12 ± 0·13	1·41 ± 0·22
MGF	1·00 ± 0·14	0·88 ± 0·13	1·20 ± 0·18
NTF-3	1·00 ± 0·35	0·99 ± 0·23	1·39 ± 0·40
MSTN	1·00 ± 0·15	1·17 ± 0·11	0·96 ± 0·08

All data are expressed as fold change (mean ± SEM, n = 8–10 subjects per target). Abbreviations for each gene are in the methods section of the text. HKG1, housekeeping gene 1; HKG2, housekeeping gene 2. Primer design notes. MGF, B2M and 28S primers were from (Roberts *et al.*, 2010) and MSTN primers were from (Dalbo *et al.*, 2011). All other primers were designed using Primer3Plus as described in the methods.

and p-rps6 (Ser235/236)], mTOR inhibition [p-PRAS40 (Thr246)], cellular energy sensing [p-AMPKα (Thr172)], mitogen-activated kinase signalling (p-ERK1/2 (Thr202/Tyr204), p-JNK (Thr183/185) and p-p38 (Thr180/Tyr182)], apoptosis [p-Bad (Ser112), p-HSP27 (Ser78), PARP (Asp214), p-p53 (Ser15) and caspase 3 (Asp175)], Jak-Stat inflammatory signalling [p-Stat1 (Tyr701) and p-Stat3 (Tyr705)] and cell survival/anti-apoptosis [p-GSK-3β (Ser9)]. Peer-reviewed journal articles for these signalling targets can be found on the manufacturer's website (www.cellsignal.com; query product #7323).

Briefly, 100 μl of muscle tissue homogenate was assayed per sample per manufacturer's instructions. Slides were developed using an enhanced chemiluminescent reagent provided by the kit, and spot densitometry was performed through the use of a UVP Imager and associated densitometry software (UVP, LLC, Upland, CA, USA). The calculation of each target was as follows: density value of the target − negative control)/summation of all density values for the sample. A representative image of the phosphoarray showing markers at the three sampling time points (PRE, 1 and 4 h post-EPC) as well as a key for the phosphoarray is shown in Fig. 1.

Statistics

Each time point for all genes explored was examined using the Shapiro–Wilk test for normality. Only 1 of 45 data sets (2·2%) was found to violate the assumption of normality [pJNK (Thr183/Tyr185) 1 h post-EPC]. Thus, a repeated-measures analysis of variance (ANOVA) utilizing within subject contrasts was used to determine whether a main effect of time was observed. When a main effect of time was observed, *post hoc* analysis was conducted using paired Student's t-test to determine whether EPC significantly altered a given biomarker at the 1 h and 4 h time points compared to each other (1 h versus 4 h) and compared to baseline (versus PRE). To correct for multiple comparisons, a Bonferroni correction was applied. To maintain a family wise error rate of 0·05, alpha was adjusted for 3 comparisons ($\alpha = 0{\cdot}05/3 = 0{\cdot}0167$). Effect sizes (ES) were also investigated using Cohen's guidelines of small, moderate, and large effects with ES values of 0·20, 0·50 and 0·80, respectively (Cohen, 1992) when a main effect of time was observed. Effect sizes were calculated as the ratio of the mean difference between respective post-EPC time points and PRE to the standard deviation of difference between the respective time points. An alpha level of 0·05 was adopted for statistical significance. All statistical analyses were performed using IBM SPSS Statistics 21 for Windows (Chicago, IL, USA). Data in the text results are expressed as mean percent difference between time points [95% confidence intervals], and data in Tables 1 and 2 are expressed as fold change from baseline ± standard error values.

Results

Acute EPC has no effect on select growth factor-related mRNAs

Changes in select growth factor-related mRNA expression patterns in response to a single bout of EPC are presented in Table 1. No significant main effect of time was observed for NGF ($P = 0{\cdot}289$), MGF ($P = 0{\cdot}369$), NTF-3 ($P = 0{\cdot}685$) or MSTN ($P = 0{\cdot}430$) mRNA expression patterns.

All phosphorylation and protein cleavage markers prior to (PRE), 1 h and 4 h post-EPC are presented in Table 2. Effect sizes for significant findings are presented in Fig. 2.

Effects of EPC on phosphorylated markers in the Akt-mTOR cascade

Akt-mTOR signalling substrates are the first cohort of phospho-proteins presented in Table 2. For p-rps6, a transient increase was observed as values were significantly elevated compared to PRE at 1 h post-EPC (+31% [+7%, 54%], $P = 0{\cdot}0165$) but returned to baseline 4 h post-EPC (−1%

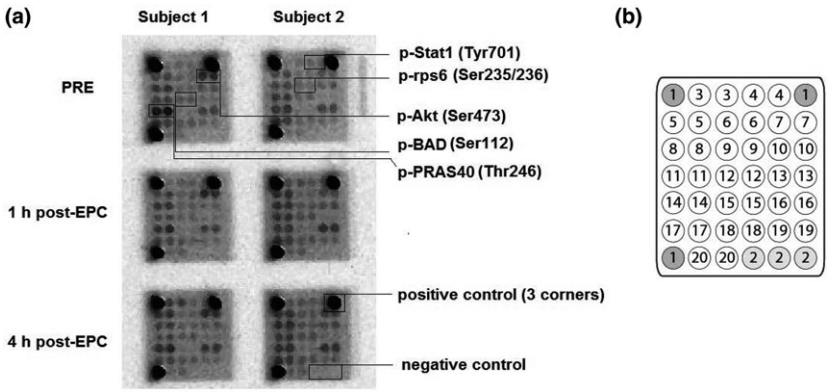

Figure 1 Representative image of phosphoarray for two subjects showing markers that changed from PRE values (panel a) as well as a key for the phosphoarray (panel b). For panel b: 1, positive control; 2, negative control; 3, phospho (p)-ERK1/2 (Thr202/Tyr204); 4, p-Stat1 (Tyr701); 5, p-Stat3 (Tyr705); 6, p-Akt (Thr308); 7, p-Akt (Ser473); 8, p-AMPKα (Thr172); 9, p-rps6 (Ser235/236); 10, p-mTOR (Ser2448); 11, p-HSP (Ser78); 12, p-BAD (Ser112); 13, p70s6k (Thr389); 14, p-PRAS40 (Thr246); 15, p-p53 (Ser15); 16, p-p38 (Thr180/Tyr182); 17, p-JNK (Thr183/Tyr185); 18, cleaved PARP (Asp214); 19, cleaved caspase-3 (Asp175); and 20, GSK-3β (Ser9).

Table 2 Effects of EPC on select phosphorylation and protein cleavage markers.

Protein	PRE	1 h post-EPC	4 h post-EPC	*P*-values		
				Main effect (time)	1 h post-EPC versus PRE	4 h post-EPC versus PRE
Akt-mTOR cascade						
p-Akt (Ser473)	1·00 ± 0·20	0·81 ± 0·12	0·46 ± 0·12	**0·048**	0·3909	0·0568
p-Akt (Thr308)	1·00 ± 0·09	0·97 ± 0·11	0·60 ± 0·16	0·073	–	–
p-mTOR (Ser2448)	1·00 ± 0·10	0·97 ± 0·10	0·86 ± 0·09	0·563	–	–
p-rps6 (Ser235/236)	1·00 ± 0·11	1·31 ± 0·07*	0·99 ± 0·14	**0·026**	**0·0165**	0·9238
p-AMPKα (Thr172)	1·00 ± 0·11	0·99 ± 0·10	0·95 ± 0·09	0·852	–	–
p-PRAS40 (Thr246)	1·00 ± 0·15	0·81 ± 0·10	0·53 ± 0·10	**0·021**	0·2632	0·0244
p-BAD (Ser112)	1·00 ± 0·07	0·88 ± 0·07	0·72 ± 0·10*	**0·002**	0·0462	**0·0040**
MAPK substrates						
p-ERK1/2 (Thr202/Tyr204)	1·00 ± 0·30	0·82 ± 0·12	0·72 ± 0·20	0·591	–	–
p-p38 (Thr180/Tyr182)	1·00 ± 0·15	0·90 ± 0·07	0·87 ± 0·09	0·627	–	–
p-JNK (Thr183/Tyr185)	1·00 ± 0·13	1·18 ± 0·10	0·99 ± 0·13	0·234	–	–
Stat signaling						
p-Stat1 (Tyr701)	1·00 ± 0·14	1·06 ± 0·15	0·62 ± 0·11†	**0·040**	0·701	0·074
p-Stat3 (Tyr705)	1·00 ± 0·09	1·27 ± 0·09	1·00 ± 0·08	0·081	–	–
Cellular stress and apoptosis markers						
p-HSP27 (Ser78)	1·00 ± 0·08	1·08 ± 0·04	0·92 ± 0·06	0·093	–	–
Cleaved caspase-3 (Asp175)	1·00 ± 0·12	1·08 ± 0·10	0·88 ± 0·12	0·482	–	–
Cleaved PARP (Asp214)	1·00 ± 0·08	0·91 ± 0·11	0·79 ± 0·14	0·156	–	–

All data are expressed as fold change (mean ± SEM, n = 8–10 subjects per target). Symbols: *, significantly different from PRE (P<0·0167); †, significantly different from 1 h (P<0.0167); values in bold are P-values that reached levels of statistical significance; Other notes: Abbreviations for each gene are in the methods section of the text. Proteins not analysed due to weak chemiluminescent signal include: p-p70s6k (Thr389), p-GSK-3β (Ser9), p-p53 (Ser15).

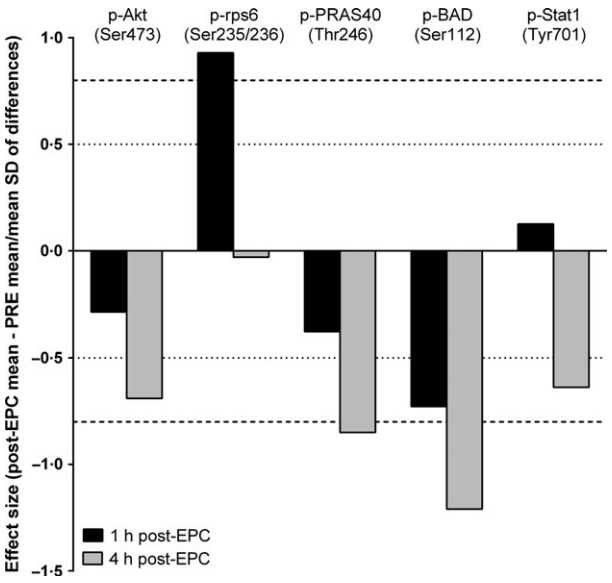

Figure 2 Effect sizes for phospho(p)-protein values in human vastus lateralis skeletal muscle biopsy samples at 1 h (black bars) and 4 h (grey bars) time points following a single 60-min treatment with peristaltic pulse external pneumatic compression (EPC). Lighter, dotted lines and darker, dashed lines demarcate moderate (±0·5) and large (±0·8) effect sizes, respectively. Effect sizes were calculated as the ratio of the post-EPC time point minus the PRE-EPC time point divided by the mean standard deviation of the between time points differences. Abbreviations for phospho-proteins are provided in the methods section of the text.

[−29%, +27%], P = 0·9238). A large effect size was also observed for p-rps6 (Ser235/236) from PRE to 1 h post-EPC values (ES = 0·930; Fig. 2). p-Bad (Ser112) values were significantly lower at 4 h post-EPC compared to PRE (−28% [−45%, −12%]; P = 0·0040), but did not reach statistical significance at 1 h post-EPC compared to PRE (−12% [−23%, −1%]; P = 0·0462). In addition, a large effect size was observed for PRE to 4 h post-EPC p-Bad (Ser112) values (ES = −1·210; Fig. 2). A main effect of time for p-Akt (Ser473) and PRAS40 (Thr246) levels was observed, but *post hoc* comparisons did not reach statistical significance. However, a large effect size was observed for the p-PRAS40 change from PRE to 4 h post-EPC (ES = −0·850; Fig. 2). No significant main effects were observed for p-Akt (Thr308), p-mTOR (Ser2448) or p-AMPKα (Thr172) in response to EPC.

MAPK signalling substrates are the second cohort of phospho-proteins presented in Table 2. No main effects of time in response to EPC were observed for p-Erk1/2 (Thr202/Tyr204), p-p38 MAPK (Thr180/Tyr182) and p- JNK (Thr183/Tyr185) levels.

Jak-Stat signalling substrates are the third cohort of phospho-proteins presented in Table 2. A main effect of time for p-Stat1 (Tyr701) values was observed, but *post hoc* tests did not reach the level of statistical significance. No main effect of time was observed for p-Stat3 (Tyr705) response to EPC.

Cellular stress and apoptosis markers are the last cohort of phospho- and cleaved proteins presented in Table 2. No main

effect of time was observed for p-HSP27 (Ser78) levels, cleaved caspase-3 (Asp175) and PARP.

Discussion

The present study is the first to explore growth factor mRNA expression as well as Akt-mTOR signalling events that occur in the lower extremity skeletal musculature following a single bout of whole limb, peristaltic pulse EPC in humans. The primary findings are that a single bout of EPC, (i) does not affect the select mRNA expression patterns of growth factors measured herein, (ii) does transiently increase phosphorylated rps6 levels 1 h post-EPC and (iii) differentially affects other phosphorylated markers in the Akt-mTOR signalling cascade; this effect being potentially affected by 4 h of fasting. While exploratory in nature, these findings warrant further investigation into the potential benefits of chronic EPC application when treatments are carried out in tandem with exercise training.

Acute EPC administration modestly increases phosphorylated rpS6 despite a decrease in the phosphorylation of multiple Akt-mTOR signalling substrates

Although little is known about how lower-limb compression effects Akt-mTOR signalling, the results from the current study suggest that EPC decreases select Akt-mTOR signalling intermediates [i.e. p-Akt (Ser473), p-PRAS40 (Thr246), p-BAD (Ser112)] with the exception of a transient 1 h post-EPC increase in p-rpS6 (Ser235/236). As can be appreciated from Fig. 2, of the significantly altered phospho-proteins, only p-rpS6 (Ser235/236) was upregulated and the observed effect size was quite large (0·93). The results obtained in the current study needs to be interpreted within the context of the experimental design. In this regard, participants in this study fasted for 4 h prior to beginning the study as well as forgoing nutrient intake for the duration of the protocol. Thus, by the end of the study, the participants were fasted for at least 9 h. Fasting has been shown to downregulate the phosphorylation of Akt and its downstream substrates (Ogata *et al.*, 2010): PRAS40 and BAD being two of these substrates. However, we did observe a modest, but transient upregulation in p-rps6 which suggests that EPC may acutely increase muscle protein synthesis. Resistance exercise is known to robustly increase rpS6 phosphorylation (Glover *et al.*, 2008), and resistance exercise with concomitant static lower-limb compression (via Kaatsu, 120 mmHg) has been shown to enhance p70s6k phosphorylation (Fujita *et al.*, 2007), as well as skeletal muscle protein synthesis (Fry *et al.*, 2010; Gundermann *et al.*, 2012). Of note, postcompression reactive hyperaemia was not shown to be linked to increased muscle protein synthesis rates and the mechanisms regarding lower-limb compression and skeletal muscle hypertrophy remains unknown. Thus, future work should pair EPC with acute and chronic exercise models in order to determine whether EPC enhances skeletal muscle hypertrophy.

It should be noted that growth factor mRNA expression patterns remained unaltered with EPC. Muscle contraction is known to transiently stimulate gene expression increases in MGF (Hameed *et al.*, 2003), NTF-3 (Sharma *et al.*, 2010) and NGF (Urai *et al.*, 2013) while decreasing MSTN (Dalbo *et al.*, 2013). Thus, our findings likely suggest that EPC is likely not a 'passive' form of muscle contraction given that these 'mechanosensitive' genes remained unaffected with EPC.

Acute EPC decreases BAD phosphorylation, but does not affect other apoptotic markers

Static compression to other tissues (e.g. Schwann cells, scar derma tissue, chondrocytes) has been shown to induce apoptosis (D'Lima *et al.*, 2001; Gupta & Steward, 2003; Reno *et al.*, 2003). Moreover, 6-h static compression (100 mmHg) for two consecutive days in rats has also been shown to induce DNA fragmentation and increase apoptotic markers (cleaved caspase-3 and increases in caspase 3/8/9 mRNA) in the compressed muscle. Authors of the aforementioned study suggested that, while the mechanisms that initiate static-pressure-induced apoptosis need more investigations, the employed prolonged static compression protocol reduced blood flow to the compressed limb by 50%, and thus, mitochondrial stress through prolonged blood flow restriction may be the initiator of apoptosis. Importantly, we report that the 1-h EPC protocol employed herein did not drastically affect select apoptotic markers [p-HSP27 (Ser78), cleaved PARP (Asp214), p-p53 (Ser15) and cleaved caspase 3 (Asp175)], although EPC modestly but significantly decreased p-Bad (Ser112) 4 h after the compression protocol; the phosphorylation of Bad at serine 112 being an anti-apoptotic index (She *et al.*, 2002). Indeed, Bad (Ser112) phosphorylation is known to be catalysed by activated MAPK substrates (Fang *et al.*, 1999). However, our data suggest that EPC did not decrease p-ERK1/2, p-JNK and/or p-p38. Thus, while the mechanisms responsible for EPC-induced Bad phosphorylation are lacking, we suggest that most blood flow restriction during the compression protocol may have been responsible for this signalling phenomenon. Notwithstanding, the lack of cleaved apoptotic substrates suggests that one bout of EPC does not lead to an 'appreciable' apoptotic insult when compared to prolonged static compression protocols.

Acute EPC stimulates Stat1 phosphorylation decreases while not affecting other cellular stress markers

We report p-Stat1 (Tyr701) to be downregulated significantly at 4 h post-EPC compared to 1 h post-EPC with a strong tendency for also being lower than PRE ($P = 0{\cdot}074$). Over 40 cytokine receptors phosphorylate STATs, and STAT activation is either pro- or anti-inflammatory (Akira, 1999). An increase in phosphorylated Stat1 has been implicated in cytokine signalling, although an increase in Stat1 phosphorylation may have a deleterious role with regard to an increase in inflammation (Hong *et al.*, 2002). Hence, an EPC-mediated decrease in p-Stat1 may

have a beneficial effect with regards to decreasing inflammation. Indeed, we recently reported that EPC tended to upregulate the mRNA expression of the anti-inflammatory cytokine IL-10 4 h post-EPC (Kephart *et al.*, 2015), an effect which may be related to a decrease in the phosphorylation state of this pro-inflammatory marker, Stat1. However, and as mentioned above regarding the design of this study, the 4 h decrement in Stat1 phosphorylation may be due to the prolonged fasting of the subjects versus a true experimental effect, albeit p-Stat3 remained unaffected by the 4 h post-EPC time point which further supports an experimental effect. Notwithstanding, the potential for EPC to transiently decrease inflammatory mediators while increasing anti-inflammatory gene expression patterns makes it attractive to test in chronically trained athletes whereby chronic low-grade inflammation during tenuous training periods can lead to 'over-training-like' states (Xiao *et al.*, 2012).

Other cellular stress markers assayed herein included phosphorylated JNK, p38 MAPK and HSP27. JNK activation in skeletal muscle can signify inflammation (Hommelberg *et al.*, 2010), and p38 MAPK activation in skeletal muscle, like JNK, has been linked to inflammation and an increase in pro-inflammatory cytokine expression (Brown *et al.*, 2015). Moreover, HSP27 is a downstream substrate of p38 MAPK and, when phosphorylated, leads to a decrease in its function as a molecular chaperone (Rogalla *et al.*, 1999). Hence, given that these markers remained unaltered with EPC, our data further support that maladaptive signalling mechanisms are not acutely activated with one bout of EPC application.

Limitations and conclusions

Indeed, there are limitations to the current study. One limitation herein is that the EPC treatment was performed in a controlled laboratory setting (i.e. subjects were fasting and physical activity post-EPC was limited) which is dissimilar to daily living conditions. Therefore, while select Akt-mTOR signalling mediators decreased 4 h following EPC, we contend that this may have been due to fasting rather than EPC treatment. In addition, the majority of participants in this study were male (80%) and the study was not designed to address differential responses between sexes. However, the observed responses amongst the female participants were not identified as outliers. Regardless, the ability of the present investigation to differentiate between-sexes responses is inadequate. Also, due to limited sample availability, we opted to perform an exploratory signalling assay utilizing a high throughput antibody chip array as opposed to individual Western blots. However, in an effort to decrease variability due to time and sampling sites, phospho-protein signals were normalized to total chip intensity. Regardless, future investigations using Western blotting and immunohistochemistry should be performed to confirm these array findings. Finally, given the exploratory nature of this investigation, 15 unique markers were evaluated and the resultant number of statistical tests increases the probability of spurious significance. Although a more conservative approach was applied for post hoc testing (Bonferroni correction), an alpha level of 0·05 was employed to determine the main effect of time for each marker. Thus, the possibility of false significance(s) cannot be excluded. In consideration of this limitation, effect sizes were also included to further illustrate the impact of EPC on variables of interest.

In spite of these limitations, these preliminary data suggest that EPC transiently increases rps6 phosphorylation, while also decreasing Stat1 phosphorylation. Given that these signalling events may facilitate positive adaptive responses to exercise, future research should investigate combined interactions of various acute and chronic exercise-related stimuli with EPC, as our data suggest that a select number of recovery parameters may be enhanced. Importantly, maladaptive cellular stress signals are not altered within a 4-h time window following EPC suggesting that it is relatively safe to implement in a young, healthy population.

Acknowledgments

The authors wish to thank the participants for devoting their time and muscle specimens to this study. In addition, the authors would like to thank NormaTec (Newton Center, MA) for their partial financial support of this project through a contract awarded to J.S.M.

Funding

Reagent costs and participant compensation costs were partially paid through a contract awarded to J.S.M. through NormaTec (Newton Center, MA).

Conflicts of interest

The authors have no conflicts of interest.

Disclosure

None of the authors have any non-finacial and/or financial competing interests to disclose.

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