# A single bout of whole-leg, peristaltic pulse external pneumatic compression upregulates PGC-1α mRNA and eNOS protein in human skeletal muscle tissue

**Running title:** Compression increases PGC-1α gene expression in human muscle biopsies

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## NEW FINDINGS

- **What is the central question of this study?**
  Does 60 minutes of peristaltic pulse external pneumatic compression (EPC) alter gene and protein expression patterns related to metabolism, vascular biology, redox balance and inflammation in *vastus lateralis* biopsy samples?

- **What is the main finding and its importance?**
  A single bout of EPC transiently up-regulates PGC-1α mRNA, while also up-regulating eNOS protein and nitric oxide metabolite concentrations in *vastus lateralis* biopsy samples.


## ABSTRACT

We investigated if a single 60 min bout of whole leg, lower pressure external pneumatic compression (EPC) altered select vascular, metabolic, anti-oxidant and inflammation-related mRNAs. Ten participants (8 male, 2 female; aged 22.0±0.4 years) reported to the laboratory 4 h post-prandial and *vastus lateralis* muscle biopsies were obtained prior to (PRE), 1 h and 4 h post-EPC treatment. mRNA expression was analyzed using real-time RT-PCR and significant mRNA findings were further investigated via Western blot analysis of respective protein concentrations. PGC-1α mRNA increased 77% 1 h following EPC compared to PRE levels ($P$=0.005), but no change in protein concentration 1 or 4 h post-EPC was observed. Increases in eNOS mRNA (+44%) and SOD2 mRNA (+57%) 1 h post-EPC as well as an increase in IL-10 mRNA (+132%) 4 h post-EPC compared to PRE levels were observed, but only approached significance ($P$=0.076, 0.077 and 0.074, respectively). Interestingly, eNOS protein (+40%, $P$=0.025) and nitrate/nitrate (NOx) concentrations (+69%, $P$=0.025) increased 1-to-4 h post-EPC. Moreover, SOD2 protein tended to increase PRE-to-4 h post-EPC (+43%, $P$=0.074) though no changes in tissue 4-hydroxnonenal levels was observed. An acute bout of EPC transiently up-regulates PGC-1α mRNA, while also up-regulating eNOS protein and NOx concentrations in *vastsus lateralis* biopsy samples. Future research should characterize the origin of these responses (e.g., vascular or muscle fiber cells) and how the


 

acute effects of EPC application on gene and protein expression observed herein are associated with functional improvements (e.g. metabolism, vascular function) in acute and chronic models.

**INTRODUCTION**

Lower limb compression therapy has been used in research and practitioner settings to alter various facets of vascular and skeletal muscle physiology. One such compressive therapy, enhanced external counterpulsation (EECP), is a high pressure-driven treatment (target inflation pressure of ~300 mmHg) applied to the lower limbs with sequential, distal-to-proximal inflation occurring during diastole and rapid cuff deflation prior to cardiac systole. Given that antegrade brachial and retrograde popliteal artery blood flow shear stress are increased 75 and 402%, respectively, during EECP administration (Gurovich & Braith, 2013), considerable focus has been on vascular adaptations. Indeed, EECP elicits marked beneficial effects on vascular biology as application has been shown to improve peripheral conduit and resistance vessel reactivity, capillary density, and circulating markers of inflammation, redox balance and vasoactive balance in clinical populations (Braith *et al.*, 2010; Martin *et al.*, 2012, 2014; Martin & Braith, 2012; Beck *et al.*, 2014). However, the benefits of EECP appear to extend beyond vascular function as we have recently shown that glycemic control is improved and skeletal muscle GLUT-4 protein expression is increased following 35 1 h sessions of EECP therapy (Martin *et al.*, 2012).

Investigations into the effects of lower pressure external pneumatic compression (EPC) devices that operate asynchronously with the cardiac cycle and at markedly lower pressures (e.g. 60-120 mmHg) include intermittent pneumatic compression (IPC). IPC has been shown to acutely impact lower limb hemodynamics, but have little effect on vascular function (Sheldon *et al.*, 2012). However, in a rodent model of IPC with target inflation pressures of 55 mmHg, eNOS protein expression has been shown to increase 1 h post-IPC application in both the compressed and upstream tissue (Tan *et al.*, 2006). Moreover, in

models of IPC with target inflation pressures of 120 mmHg, skeletal muscle mRNA expression of VEGF, MCP-1, and PGC-1α were increased and IL-6 decreased following a single treatment (Roseguini *et al.*, 2010; Sheldon *et al.*, 2012). Collectively, these investigations demonstrate that lower pressure EPC significantly acutely alters skeletal muscle gene expression patterns. Importantly, the latter IPC investigations (Roseguini *et al.*, 2010; Sheldon *et al.*, 2012) were vital in establishing that a higher frequency of compression, and not magnitude (i.e. target inflation pressure), was a primary factor potentiating the gene expression response.

Lower pressure EPC is favorable as it potentiates independent, at-home usage. IPC, a form of lower pressure EPC, is a single cell chamber that is applied to only the foot/calf area. However, an alternative lower pressure EPC device, sequential peristaltic pulse EPC, is commercially available. Given that this form of EPC employs higher frequency compressions and encompasses a greater volume of the lower limbs, it may be a superior stimulus to IPC and elicit similar responses to those observed with EECP. Indeed, preliminary studies have demonstrated that this form of EPC has been shown to improve vascular reactivity in the peripheral conduit (i.e. flow mediated dilation) and resistance (i.e. peak reactive hyperemic blood flow) arteries following a single 60 min bout of sequential peristaltic pulse EPC at ~70 mmHg (Martin & Laughlin, 2013; Martin & Borges, 2015). However, the acute effect of this device on select gene and protein expression markers in skeletal muscle tissue samples has not been characterized. Therefore, the purpose of this study was to determine if an acute bout of EPC affects select mRNA markers related to vascular function, metabolism, inflammation, and oxidative stress in human *vastus lateralis* biopsy samples. While exploratory in nature, a chief outcome of this study is to guide future areas of mechanistic and applied research using EPC devices.

## 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. In addition, written informed consent was obtained from all participants prior to their voluntary participation in the study.

*Participants*

Ten college-aged volunteers were enrolled in this study (n = 8 males, n = 2 females). Eligibility criteria included the following: a) participants were apparently healthy, medication-free and had no history of blood clotting issues; and b) participants had not consumed ergogenic nutritional supplements for at least 1 month prior to the compression bout. Participants also filled out a short questionnaire indicating their self-reported amounts of weekly physical activity. Participant characteristics are presented in Table 1.

INSERT TABLE 1 HERE

*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. Participants were then instructed to lay in a supine position on an athletic training 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 (Evans *et al.*, 1982; Roberts *et al.*, 2010; Dalbo *et al.*, 2013). 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 1x 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 -80ºC.

Thereafter, 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 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 that we have previously investigated and described (Martin *et al.*, 2015). In brief, beginning with the most distal chamber, inflation occurs and the chamber "pulses" (slight variations in pressure) for ~1 min 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 is 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 approximately 30 seconds. This entire cycle of compression is repeated continuously over the course of a single 60 minute treatment session.

After the 60 min EPC protocol, the legging was removed. 1 and 4 h following EPC, two separate skeletal muscle biopsies, spaced 5 cm apart, were obtained from the right *vastus lateralis* in order 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 signaling 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 EPC and 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 compression bout in order to control for physical activity levels during the trial.

*RNA isolation and mRNA analyses*

10-20 mg portions of muscle biopsy samples were homogenized in 500 µl of Ribozol (Ameresco) and stored at -80ºC 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. 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 2). The following mRNAs were analyzed: peroxisome proliferator-

activated receptor gamma co-activator-1alpha (PGC-1α), sirtuin-1 (SIRT-1), TBC1 domain family, member 4 (TBC1D4), glucose transporter-4 (GLUT-4), hypoxia inducible factor 1, alpha subunit (HIF-1α), vascular endothelial growth factor A isoform 165 ($VEGF_{165}$), vascular endothelial growth factor A isoform 189 ($VEGF_{189}$), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RN), superoxide dismutase 2 (SOD2), catalase (CAT), glutathione reductase (GSR), glutathione peroxidase (GPx), endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). 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.

INSERT TABLE 2 HERE

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), where $-\Delta\Delta CT$ = (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 reaction for each gene, melt curve analyses were performed to ensure that one PCR product was amplified per reaction. Primer sequences are presented in Table 2.

*Protein isolation & Western blotting*

As mentioned previously, approximately 50 mg of muscle was placed in a 1.7 ml polypropylene tube containing 500 µl of cell lysis buffer (Cell Signaling). Immediately following muscle extraction, samples were homogenized using a tight-fitting micropestle, insoluble proteins were removed with centrifugation at 500 x *g* for 5 min at 4°C, and supernatants containing muscle tissue homogenate were collected and stored at -80ºC. After all participants finished the study, muscle tissue homogenates were batch-assayed for total protein content using a BCA Protein Assay Kit (Thermo Scientific) and were diluted to 1.0 µg/µl using additional cell lysis buffer (Cell Signaling).

Cell lysis homogenates obtained from above were prepared for Western blotting using 4x 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 pre-made 1x 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. Rabbit anti-4HNE IgG (1:1,000; Abcam, Cambridge, MA USA), anti-eNOS IgG (1:500; Cell Signaling), anti-PGC-1α (1:1,000; Santa Cruz, Dallas, TX, USA), anti-HIF-1α (1:1,000; Santa Cruz) and anti-SOD2 (1:1,000; Santa Cruz) were incubated with membranes overnight at 4º C in 5% bovine serum albumin (BSA), and the following day membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000, Cell Signaling) at room temperature for 1 h prior to membrane development. Membrane development 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 4HNE, eNOS, PGC-1α, and SOD2 were normalized to Ponceau densities, and these values were normalized to PRE values in order to obtain fold-change values from 1.00.

*Intramuscular NOx determination*

Cell lysis homogenates obtained from above were prepared to estimate nitric oxide production via measurement of the stable metabolites nitrite and nitate (NOx) using a spectrophotometric kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions. Of note, cell lysis buffer only wells were assayed and no interfering reagents were found to exist.

*Statistics*

A repeated measures analysis of variance (ANOVA) utilizing within subject contrasts were used to determine if a main effect of time was observed with primary continuous outcome variables in response to a single, 60 min bout of EPC. Effect sizes were also investigated using Cohen's guidelines of small, medium, and large effects with partial eta squared ($\eta_p^2$) values of 0.01, 0.09, and 0.25, respectively (Parker & Hagan-Burke, 2007). *Post hoc* analysis of between time points comparisons were conducted when a significant main effect of time and/or a large effect size ($\eta_p^2 \geq 0.25$) was observed using Student's paired-tests. 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). All data are presented in as means ± standard error values.

**RESULTS**

The effects of EPC on select 1 h and 4 h post-treatment mRNA expression patterns are presented in Table 3.

INSERT TABLE 3 HERE

*Acute EPC administration transiently increases PGC-1α*

In response to a single 60-min bout of EPC, a main effect of time for PGC-1α mRNA expression was observed ($P$=0.026). *Post hoc* analysis revealed a transient up-regulation of PGC-1α mRNA as the 1 h post-EPC time point was significantly greater than PRE (+77%, $P$=0.005), but was no longer significantly greater at the 4 h post-EPC time point (+33%, $P$=0.411; Figure 1A). Western blot analysis of PGC-1α protein expression revealed no main effect of time ($P$=0.766; Figure 1B). With regards to the other selected metabolic-related mRNAs examined, no significant main effect of time was observed for SIRT-1 mRNA ($P$=0.683), TBC1D4 mRNA ($P$=0.389), or GLUT-4 mRNA ($P$=0.315).

INSERT FIGURE 1 HERE

*EPC decreases HIF-1mRNA 4 h following a single treatment*

A significant main effect of time for HIF-1α mRNA response to EPC was observed ($P$=0.049), with *post-hoc* analysis revealing a significant 20% decrease relative to PRE at the 4 h post-EPC time point ($P$=0.049; Figure 2A). HIF-1α protein expression was not detected with the antibody selected during Western blot analysis. With regards to other select angiogenic-related genes explored herein, no main effect of time was observed for$VEGF_{189}$ mRNA ($P$=0.960) and $VEGF_{165}$ mRNA expression patterns ($P$= 0.492).

*Acute EPC modestly elevates anti-inflammatory IL-10 mRNA with little effect on pro-inflammatory genes*

For pro-inflammatory genes, a main effect of time was not observed for IL-6 mRNA (*P*= 0.613), TNF-α mRNA (*P*= 0.618), or IL1-β mRNA expression patterns (*P*=0.170) following EPC. Changes in IL1-β mRNA with time revealed a large effect size ($\eta_p^2$=0.250), but *post hoc* analysis failed to reveal any significant difference between any of the time points.

Regarding select anti-inflammatory markers, although there was no significant main effect of time (*P*= 0.102), a large effect size ($\eta_p^2$=0.298) was observed for IL-10 mRNA . *Post-hoc* analysis revealed that IL-10 mRNA tended to be up-regulated (+132%) compared to PRE at the 4 h time point, but did not reach statistical significance (*P*=0.074, Figure 2B). Due to limited sample size, protein expression analysis was not performed for IL-10. No main effect of time was observed for IL1-RN (*P*=0.195).

INSERT FIGURE 2 HERE

*EPC increases SOD2 gene expression with little alteration to other select redox markers*

No main effects of time existed for CAT mRNA (*P*=0.314) and GSR mRNA expression patterns (*P*=0.419) and GPx mRNA was not detected using the designed primer. SOD2 mRNA expression patterns revealed a significant main effect of time (*P*=0.042), and *post hoc* analysis revealed a tendency for greater mRNA expression at 1 h post-EPC compared to PRE (+57%, *P*=0.077; Figure 3A). For SOD2 protein content responses to EPC, a large effect size ($\eta_p^2$=0.420) and a tendency for a main effect of time (*P*=0.070) was observed. *Post-hoc* analysis revealed that relative to PRE, SOD2 protein tended to be higher

at 4 h post-EPC (+43%), but did not reach statistical significance ($P$=0.074; Figure 3B). Finally, no main effects of time existed for 4HNE-labelled proteins ($P$=0.958, Figure 3D).

INSERT FIGURE 3 HERE

*Acute EPC modestly increases eNOS gene expression, eNOS protein concentration and NOX concentrations*

A significant main effect of time was not observed for nNOS mRNA expression ($P$=0.455) and the iNOS mRNA expression was not detected using the designed primer. Analysis of eNOS mRNA expression did not reveal a significant main effect of time ($P$=0.165) though a large effect size was observed ($\eta_p^2$=0.330). *Post hoc* analysis indicated that eNOS mRNA expression was transiently up regulated by 44% at the 1 h time point, but only approached statistical significance compared to PRE ($P$=0.076, Figure 4A). Given the eNOS mRNA findings, we also explored eNOS protein content and intramuscular NOx levels in response to acute EPC administration. A significant main effect of time was observed for eNOS protein expression ($P$=0.016) following EPC and *post-hoc* analysis revealed that the 36% increase 4 h post-EPC tended to be higher than PRE ($P$=0.075) and was significantly higher than 1 h post-EPC ($P$=0.025; Figure 4C). For post-EPC intramuscular NOx there tended to be a main effect of time with a large effect size ($P$=0.082, $\eta_p^2$=0.420) and *post-hoc* analysis revealed a significant 99% increase at 4 h post-EPC relative to 1 h post-EPC ($P$=0.025), but not relative to PRE (+55%, $P$=0.210; Figure 4B).

INSERT FIGURE 4 HERE

## DISCUSSION

The primary findings of this study are that a single bout of peristaltic pulse EPC transiently upregulates PGC-1α mRNA expression and also alters eNOS protein and NOx concentrations in human *vastus lateralis* biopsy samples. However, our current data make it difficult to determine the localization of EPC-mediated mRNA and protein changes; specifically, muscle biopsy samples contain mature muscle cells, satellite cells, resident immune cells and mirco-vessels. Notwithstanding, the present study is the first to explore acute changes in gene expression and protein content that occur in the lower extremity skeletal musculature following a single EPC treatment in humans. Herein, we demonstrate that a single bout of relatively lower pressure, peristaltic pulse EPC elicits similar changes in PGC1-α mRNA and eNOS mRNA as those elicited by IPC in humans. Moreover, we demonstrate, for the first time, increases in eNOS protein and NOx concentrations, and a tendency for increased SOD2 protein concentrations in human skeletal muscle biopsy samples following an acute whole leg EPC treatment. While exploratory in nature, these findings warrant investigation into the mechanisms for EPC-mediated responses and the benefits of chronic application in healthy and diseased populations given that PGC-1α is a regulator of tissue metabolism and angiogenesis (Kim *et al.*, 2007; Olesen *et al.*, 2010). In addition, our data demonstrating modulation of eNOS protein and NO metabolite concentrations in skeletal muscle biopsy samples is intriguing for future investigations employing EPC in populations with microvascular dysfunction.

*Acute administration of EPC transiently upregulates PGC-1α gene expression in human vastus lateralis biopsy samples*

PGC-1α is a well know activator of tissue metabolism (Wu *et al.*, 1999), and is also central to skeletal muscle adaptation following exercise (Baar *et al.*, 2002; Olesen *et al.*, 2010). Here we present novel evidence that a single bout of whole leg, peristaltic EPC significantly upregulates PGC-1α gene expression (77%) in human *vastus lateralis* biopsy samples 1 h following application (Figure 1A). Our findings are similar to those observed by Sheldon et al. (Sheldon *et al.*, 2012) who demonstrated significant up-regulation in PGC-1α mRNA (~50%) in lateral gastrocnemius biopsy samples 30 min following an acute bout of IPC in humans. However, those changes were observed in the lower aspect of the leg following a more static model of compression with a markedly different duty cycle. Therefore, it appears the low-pressure EPC protocol employed in the current study is also able to facilitate an acute increase in PGC-1α mRNA, and that this phenomena extends to biopsy samples of the larger proximal muscles of the quadriceps (i.e. *vastus lateralis*). However, future experiments should determine if direct compression of the sampled tissue is required for this EPC-mediated increase in PGC-1α, or if upstream tissues (e.g. *vastus lateralis*) would respond similarly to partial leg compressive therapies such as IPC.

Although we did not observe any change in PGC-1α protein expression following EPC, this was not surprising given that increased PGC-1α protein content is not observed until approximately 24 hours post stimulus even with intense exercise stimuli (Little *et al.*, 2011) and protein concentrations may have changed at a point beyond the 4 h post-EPC sampling time point measured in this study. Various signals (i.e., calcium, AMPK, reactive oxygen species, and beta-adrenergic signaling) and substrate availability appear to be able to regulate PGC-1α signaling (Olesen *et al.*, 2010). Although signaling in these pathways was not a focus of the present investigation, we did observe modest increases in mRNA (SOD2,

eNOS), protein (SOD2, eNOS) and metabolite (NOx) concentrations associated with redox signaling in the *vastus lateralis* biopsy samples. However, no change in 4HNE labeled protein was observed at any point post-EPC. Although it is possible that oxidative stress was acutely and transiently elevated during EPC application and not captured by our sampling time points, SOD2, eNOS and NO production are all known to be upregulated by blood flow shear stress (Topper & Gimbrone Jr, 1999) and IPC has been shown to significantly increase hemodynamic shear stress during treatment (Roseguini *et al.*, 2010; Sheldon *et al.*, 2012). Given that EPC is also likely to cause perturbations in the local resistance vasculature during treatment, and that PGC-1α is expressed in endothelial cells (Valle *et al.*, 2005; Kim *et al.*, 2007), it is reasonable to hypothesize that EPC-mediated alterations of hemodynamics are, at least, partially responsible for the transient upregulation PGC-1α observed herein. However, due to limited sample amounts we were not able to evaluate potential effects of EPC on localized responses or the translocation of PGC-1α into the nucleus, but this would be of interest in future investigations characterizing the effects of EPC on PGC-1α and its activation. Thus, our current data precludes us from comprehending the physiological relevance in the transient EPC-mediated alteration in PGC-1α mRNA expression, and more research is needed to determine: a) the localization of this response; b) the EPC-initiated signaling pathway that facilitates this mRNA response; and c) a more thorough post-EPC time-course.

*Acute application of EPC does not alter select angiogenic markers in human vastus lateralis biopsy samples*

Select angiogenic markers (mRNAs for $VEGF_{189}$, and $VEGF_{165}$) were minimally altered 1 h and 4 h post-EPC compared to baseline levels. In addition, we observed a modest, though statistically significant, decrease in HIF-1α mRNA expression 4 h post-EPC relative to PRE (Figure 2A). These findings are somewhat in discord with previous findings, as Roseguini et al. (Roseguini *et al.*, 2010) reported an up-regulation of skeletal muscle VEGF mRNA utilizing IPC in rodents. One potential explanation for this discordant finding could be a difference in protocols utilized. The IPC employed by Roseguini et al. utilized higher pressures (120 mm Hg) compared to those presently tested EPC (~70 mm Hg) and the duty cycles are fundamentally different. Indeed, the EPC protocol employed herein applied sequential, peristaltic pulse compression linearly along the leg with multiple (five) inflation chambers. Therefore, compression is intermittently distributed along the legs as opposed to focusing on a single zone of the lower limb. Secondly, the timing of tissue sampling also differed between studies as Roseguini et al. noted a significant increase in VEGF mRNA immediately post-IPC, whereas our earliest sampling point was 1 h post-EPC. Thus, it is possible that alteration of these angiogenic signals with EPC were transient in nature and not captured with the measurement of mRNA expression 1 and 4 h post treatment. Finally, the possibility of inter-species differences exists. Nonetheless, acute EPC application has been shown to elicit improvements in vascular function (Martin & Laughlin, 2013; Martin & Borges, 2015), likely mediated, in part, through alteration of hemodynamic signals and this warrants future research regarding the effects of chronic EPC administration with varying target pressures on angiogenic indices.

*EPC may alter anti-inflammatory gene expression in skeletal muscle tissue*

The IPC protocol in rodents employed by Roseguini et al. up-regulated pro-inflammatory MCP-1 mRNA in skeletal muscle immediately post-application (Roseguini *et al.*, 2010). Herein, we observed no changes in select pro-inflammatory genes, and observed a trend for increased expression of the anti-inflammatory gene IL-10 4 h post EPC treatment (Figure 2B). However, variability among IL-10 mRNA expression responses was high, and we were not able to characterize IL-10 protein content changes post-EPC. Regardless, our findings are dissimilar from those previously reported but could be due to the aforementioned differences in protocols utilized, tissue sampling timing, and/or species studied. Chronic EECP treatment in humans has been shown to decrease circulating levels of pro-inflammatory mediators in humans with abnormal glucose tolerance (Martin & Braith, 2012). However, EECP utilizes much greater pressures (~300 mm Hg), and the acute study design employed herein makes these inter-study comparisons difficult to interpret. Notwithstanding, our findings suggest that acute EPC application may favorably alter anti-inflammatory signaling while not affecting pro-inflammatory gene expression patterns. Alternatively, given that cytokine mRNAs can be produced by immune cells, the IL-10 response may have been driven to a good extent by EPC-mediated increases in blood flow which, in turn, recruited more leukocytes to the skeletal muscle tissue bed. Therefore, more acute mechanistic studies are needed to investigate if EPC-mediated alterations in cytokine mRNAs are due to an increase in tissue leukocytes. Moreover, if EPC is indeed anti-inflammatory, chronic interventions are needed in order to examine if this facet is beneficial for both athletic and clinical populations.

*Acute EPC may stimulate an up-regulation in SOD2 mRNA and protein expression in the absence of oxidative stress in human skeletal muscle tissue*

EPC tended to increase SOD2 mRNA expression (+57%) 1 h post-treatment and returned back to baseline levels 4 h post-treatment (Figure 3A). Moreover, these changes were associated with a 43% increase in SOD2 protein content 4 h post-EPC (Figure 3B). The SOD2 enzyme reduces superoxide anion levels to prevent cellular damage (Zelko *et al.*, 2002), and an increase in oxidative stress (e.g., prolonged exercise or ischemia-re-perfusion) may initiate SOD2 gene expression in a compensatory manner (Powers & Jackson, 2008). Thus, we initially hypothesized that the modest up-regulation in SOD2 mRNA expression and protein content may have been due to an EPC-induced increase in oxidative stress. Upon discovering that 4HNE levels in the muscle remained unaltered up to 4 h post-EPC application (Figure 3D), we posit that mechanical strain and/or altered microvascular blood flow profiles produced during EPC may have increased SOD2 mRNA levels. Indeed, mechanical strain of myotubes has been shown to up-regulate SOD2 gene expression (Pardo *et al.*, 2011) and blood flow shear stress is a well-known signal for upregulation of SOD2 in the vasculature (Topper & Gimbrone Jr, 1999). However, given that SOD2 mRNA increases only approached statistical significance, speculating as to the mechanisms responsible for this observation is tenuous. Moreover, the localization of these changes (e.g., in the mitochondria of skeletal muscle versus micro-vessels) remains speculative given that we lacked tissue to further interrogate this marker with immunohistochemistry.

*Acute administration of EPC modestly increases skeletal muscle tissue eNOS protein and NOx concentrations*

Interestingly, we report that eNOS mRNA increased 44% 1 h following EPC which approached statistical significance (p=0.077; Figure 4A). In addition, this change in eNOS mRNA expression was followed by 36% and 55% increases in eNOS protein and NOx (stable metabolites of NO) in skeletal muscle tissue samples 4 h following EPC relative to PRE (Figure 4C,B). Though these increases did not reach statistical significance compared to PRE, they were significantly higher than 1 h post-EPC values. As can be appreciated from Figure 4, the 1 h post-EPC values did not change relative to PRE (within 10% of baseline) and there was considerably less variability. Given our relatively small sample size, as there was only enough complete sample sets remaining to analyze 7 subject responses, and the large effect sizes observed, our data suggests significant alteration of eNOS expression and/or NO bioavailability in the biopsied muscle tissue and should be further characterized in future investigations.

eNOS and nNOS mRNA expression has been reported to increase in the aforementioned animal models of IPC (Chen *et al.*, 2002; Tan *et al.*, 2006). The current EPC treatment failed to demonstrate a similar up-regulation of nNOS, and our elevations in eNOS mRNA were not as robust as the aforementioned reports. However, our findings are similar those observed in human models demonstrating up-regulation of eNOS mRNA following an acute bout of IPC (Sheldon *et al.*, 2012). Findings from the NOx assay along with the modest increase in eNOS mRNA and protein content in the current study suggest that a relationship could exist between an increased eNOS expression and an increase in NO production. Moreover, given that peak skeletal muscle tissue NO levels were present 4 h post-EPC it remains plausible that these levels could have continued to increase following this sampling time point. However, as with other molecular targets assayed herein, it is important to note

that the localization of EPC-mediated alterations in eNOS mRNA/protein and NOx was not interrogated. Therefore, more research is warranted regarding if: a) if skeletal muscle tissue NO production peaks at later time points following EPC; b) if eNOS mRNA and/or protein alterations occurred in skeletal muscle (to which there is a small contribution to the muscle tissue eNOS pool) or micro vessels [which largely comprises the total eNOS pool(Stamler & Meissner, 2001)]; and c) the physiological relevance of EPC-mediated muscle tissue NO increases.

*Limitations and conclusions*

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. Another constraint of this study was the population that was tested (i.e., healthy young adults). Therefore, future studies should characterize the effect of EPC on skeletal muscle signaling in populations of varying age and health. Of note, due to either a negligible sample response or sample availability for subsequent protein/NOx analyses, there was a range for number of subject samples analyzed (7-10) for all analyses. Although at least 1 female participant was included in each of the analysis presented herein, the majority of the participants were male and the study was not designed to address potential differences between sexes. Therefore, the ability of this study to speak to possible sex differences is inadequate. Regardless, these preliminary data suggest that EPC increases the mRNA expression patterns of PGC-1α, while also potentially affecting select anti-inflammatory, antioxidant, and NO related markers in skeletal muscle tissue. Finally, the pervading limitation to the current data set includes the lack of localization regarding alterations in molecular markers. Collectively, the responsive genes (e.g., eNOS, but not nNOS) suggest that the vasculature may be the predominant target for EPC-mediated

changes in skeletal muscle biopsy gene and protein expression. However, further experiments are needed to clarify which tissue types (e.g. microvasculature vs. skeletal muscle fibers) acutely respond to and/or chronically adapt to EPC. Notwithstanding, the present study is the first to explore acute changes in gene expression and protein content that occur in the lower extremity skeletal musculature following a single bout of EPC in humans, and this research will serve as a platform to guide more acute mechanistic and/or chronic efficacy models in healthy and diseased populations.

## COMPETING INTERESTS

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

## AUTHOR CONTRIBUTIONS

1) This person has made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data.

2) This person primarily was involved in drafting the manuscript or revising it critically for important intellectual content.

3) This person gave final approval of the version to be published.

4) This person agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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C.B.M.: 1, 2, 3, 4

C.D.F.: 1, 3, 4

D.D.P.: 1, 3, 4

J.M.S.: 2, 3, 4

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**Table 1.** Subject Characteristics

| Age (years) | Height (cm) | Body Mass (kg) | BMI ($kg/m^2$) | Aerobic Exercise (min/wk) | Resistance Exercise (min/wk) |
|---|---|---|---|---|---|
| 22.0 ± 0.4 | 178.1 ± 3.3 | 77.3 ± 3.1 | 24.3 ± 0.8 | 90 ± 36 | 149 ± 48 |

**Table 2.** Primer sequences for RT-PCR

| Gene | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|---|---|---|
| GLUT-4 | CTGGGCCTCACAGTGCTAC | GTCAGGCGCTTCAGACTCTT |
| PGC-1α | CAAGCCAAACCAACAACTTTATCTCT | CACACTTAAGGTGCGTTCAATAGTC |
| SIRT-1 | TACGACGAAGACGACGACGA | AAGGTTATCTCGGTACCCAATCG |
| TBC1D4 | AGAGTGCCAAGACGCAGATT | CTGGTCACTGACTGGCTTCA |
| HIF-1α | GCCCCAGATTCAGGATCAGA | TGGGACTATTAGGCTCAGGTGAAC |
| $VEGF_{165}$ | ATCTTCAAGCCATCCTGTGTGC | CAAGGCCCACAGGGATTTTC |
| $VEGF_{189}$ | ATCTTCAAGCCATCCTGTGTGC | CACAGGGAACGCTCCAGGAC |
| IL-1β | GGACAAGCTGAGGAAGATGC | TCGTTATCCCATGTGTCGAA |
| IL-6 | AAAGAGCGACTGCGAGAAAA | TTTCACCAGGCAAGTCTCCT |
| TNF-α | CCTGTGAGGAGGACGAACAT | GGTTGAGGGTGTCTGAAGGA |
| IL-1RN | CTTGGCAGGTACTCAGCGAA | AGGGAACTTTGCACCCAACA |
| IL-10 | GAAGAGAAACCAGGGAGCCC | ATCCCTCCGAGACACTGGAA |
| eNOS | CAGAAGAGCCCAGGGAACAG | GGAACCACTTCCACTCCTCG |
| iNOS | GAGGAGATGCTGGAGATGGC | ACATAGACCTTGGGCTTGCC |
| nNOS | TTTGGCAATGGAGATCCCCC | GGGGTGCCTCATTTCCATCA |
| CAT | CTGACTACGGGAGCCACATC | AGATCCGGACTGCACAAAGG |
| GSR | AAAAAGTACACCGCCCCACA | ATCTGGCTCTCATGAGGGGT |
| GPx | ACGAGGGAGGAACACCTGAT | TCTGGCAGAGACTGGGATCA |
| SOD2 | GTTGGGGTTGGCTTGGTTTC | GCCTGTTGTTCCTTGCAGTG |
| B2M (HKG1) | ATGAGTATGCCTGCCGTGTGA | GGCATCTTCAAACCTCCATG |
| 28S (HKG2) | ACCTGTCTCACGACGGTCTA | GCGTTGGATTGTTCACCCAC |

Abbreviations: gene abbreviations are in the methods section of the text; HKG1, housekeeping gene 1; HKG2, housekeeping gene 2. Primer design notes: PGC-1α primers were from (Pilegaard *et al.*, 2003). GLUT-4 primers were from (Stuart *et al.*, 2006). $VEGF_{165}$ and $VEGF_{189}$ primers were from (Medford *et al.*, 2009). B2M and 28S primers were from (Roberts *et al.*, 2010). TNF-α primers were from (Chang *et al.*, 2013). All other primers were designed using Primer3Plus as described in the methods.

**Table 3.** Effects of EPC on select mRNA expression patterns

| Gene | PRE | 1 h post EPC | 4 h post EPC |
|---|---|---|---|
| *Metabolic Genes* | | | |
| GLUT-4 | 1.00 ± 0.17 | 1.23 ± 0.20 | 0.80 ± 0.14 |
| PGC-1α | 1.00 ± 0.14 | **1.77 ± 0.23**** | 1.33 ± 0.35 |
| SIRT-1 | 1.00 ± 0.11 | 1.05 ± 0.16 | 0.94 ± 0.08 |
| TBC1D4 | 1.00 ± 0.14 | 1.16 ± 0.15 | 0.91 ± 0.07 |
| *Angiogenic-related Genes* | | | |
| HIF-1α | 1.00 ± 0.09 | 1.05 ± 0.12 | **0.80 ± 0.05*** |
| $VEGF_{165}$ | 1.00 ± 0.13 | 1.23 ± 0.21 | 0.91 ± 0.22 |
| $VEGF_{189}$ | 1.00 ± 0.19 | 1.02 ± 0.18 | 1.02 ± 0.09 |
| *Inflammation-related Genes* | | | |
| IL-1β | 1.00 ± 0.13 | 1.18 ± 0.17 | 1.38 ± 0.21 |
| IL-6 | 1.00 ± 0.19 | 0.77 ± 0.15 | 0.87 ± 0.13 |
| TNF-α | 1.00 ± 0.27 | 1.19 ± 0.30 | 1.51 ± 0.44 |
| IL1-RN | 1.00 ± 0.30 | 1.35 ± 0.29 | 2.36 ± 0.71 |
| IL-10 | 1.00 ± 0.35 | 0.94 ± 0.21 | 2.32 ± 0.66† |
| *Anti-oxidant Genes* | | | |
| CAT | 1.00 ± 0.12 | 1.26 ± 0.16 | 0.97 ± 0.09 |
| GSR | 1.00 ± 0.07 | 1.30 ± 0.17 | 1.12 ± 0.17 |
| SOD2 | 1.00 ± 0.15 | 1.57 ± 0.24† | 1.05 ± 0.12 |
| *Nitric Oxide Synthesis Genes* | | | |
| eNOS | 1.00 ± 0.09 | 1.44 ± 0.08† | 1.28 ± 0.20 |
| nNOS | 1.00 ± 0.12 | 1.24 ± 0.13 | 1.15 ± 0.17 |

All data are expressed as fold change (mean ± S.E.M., n = 8-10 subjects per target). Symbols: *, significantly different from PRE ($P< 0.05$); **, significantly different from PRE ($P< 0.01$); †, trend for significantly different from PRE ($0.05<P<0.10$). Other notes: Abbreviations for each gene are in the methods section of the text. Proteins not analyzed due to primer failure include: GPx and iNOS.

**Figure 1.** The acute effects of EPC on 1 h and 4 h post-treatment skeletal muscle peroxisome proliferator-activated receptor gamma co-activator-1alpha (PGC-1α) mRNA (panel A) and PGC-1α protein content (panel B). A representative Western blot image of PGC-1α protein levels and respective Ponceau images are presented in panel C. All data are expressed as fold-change from PRE levels (mean ± S.E.M., n = 7-10 subjects per target). Dashed lines represent individual response patterns. Significance from between time points comparisons using Student's t-tests are indicated within each panel.

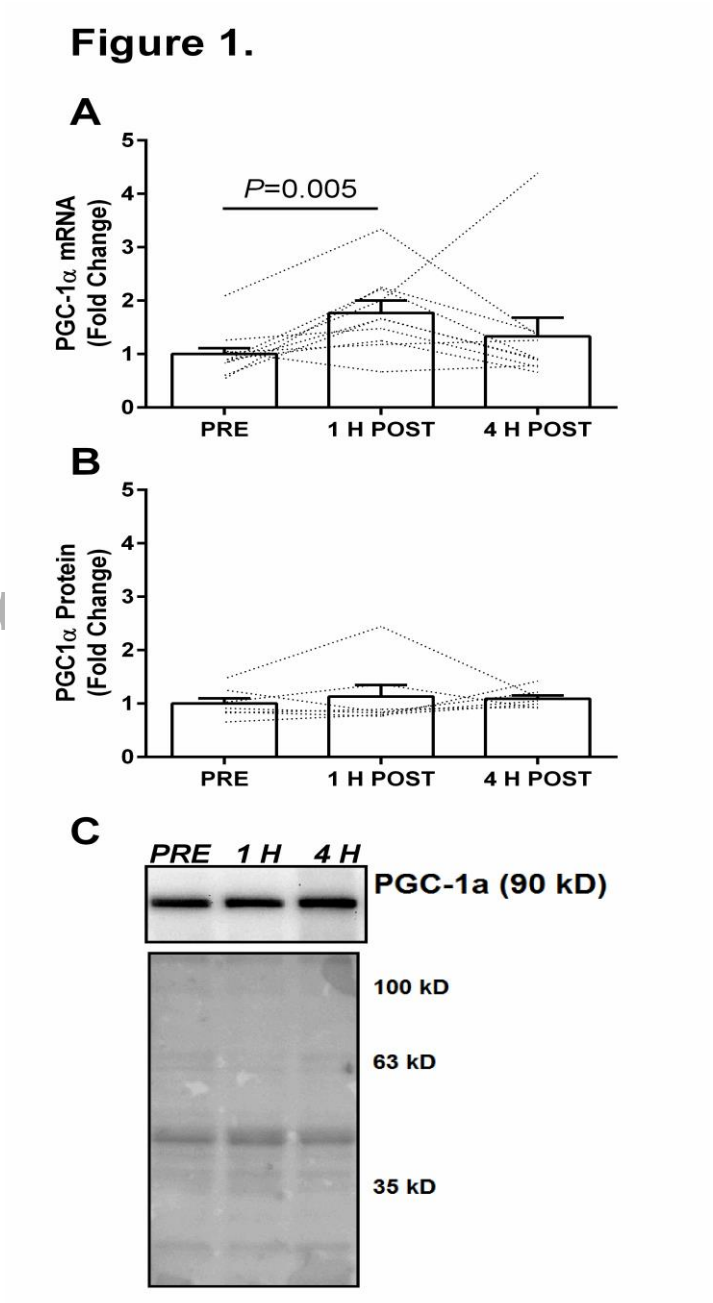

**Figure 2.** The acute effect of EPC on 1 h and 4 h post-treatment skeletal muscle hypoxia inducible factor 1, alpha subunit (HIF-1α) mRNA (panel A) and interleukin-10 (IL-10) mRNA (panel B). All data are expressed as fold-change from PRE levels (mean ± S.E.M., n = 8-10 subjects per target). Dashed lines represent individual response patterns. Significance from between time points comparisons using Student's t-tests are indicated within each panel.

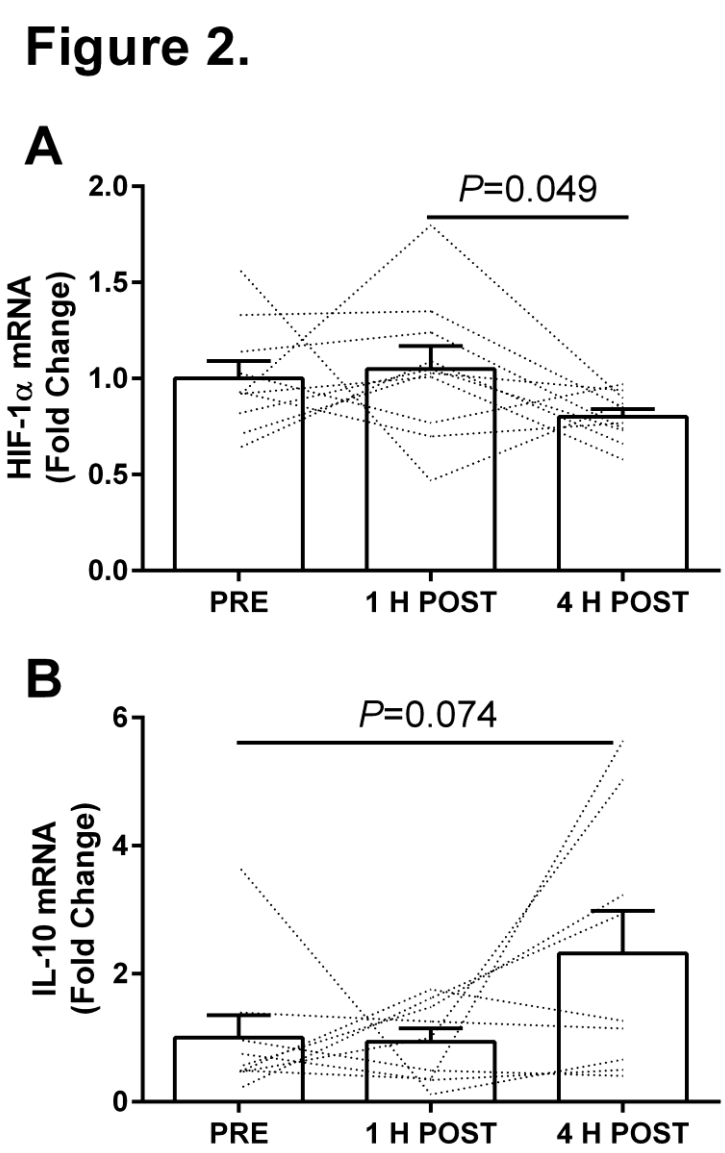

**Figure 3.** The acute effect of EPC on 1 h and 4 h post-treatment skeletal muscle endothelial nitric oxide synthase (eNOS) mRNA (panel A), concentration of the stable nitric oxide metabolites nitrate and nitrate (NOx; panel B), and eNOS protein content (panel C). A representative image of eNOS protein levels and respective Ponceau images are presented in panel D. All data are expressed as fold-change from PRE levels (mean ± S.E.M., n = 7-10 subjects per target). Dashed lines represent individual response patterns. Significance from between time points comparisons using Student's t-tests are indicated within each panel.

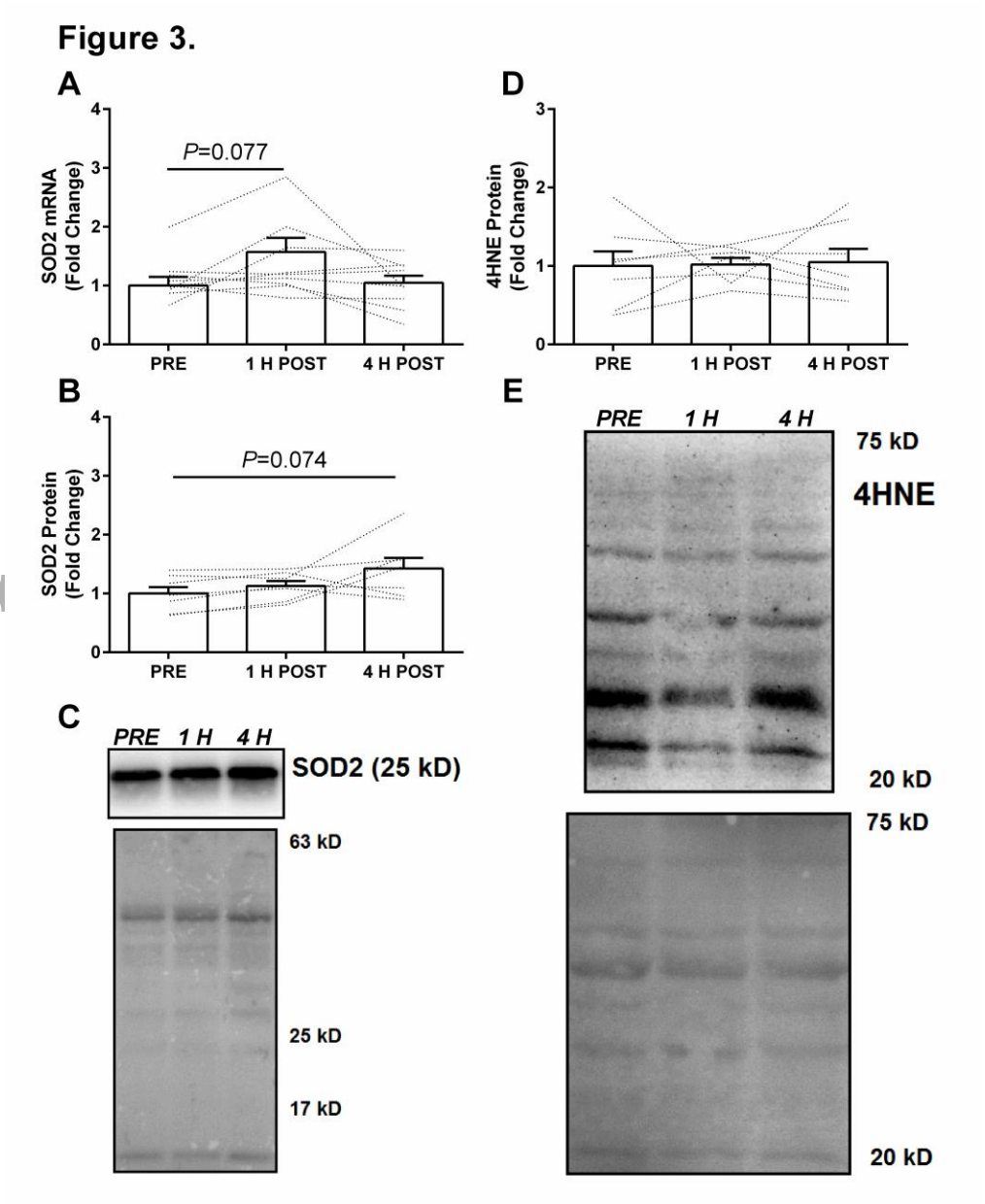

**Figure 4.** The acute effect of EPC on 1 h and 4 h post-treatment skeletal muscle superoxide dismutase 2 (SOD2) mRNA (panel A), SOD2 protein content (panel B), and 4-hydroxynonenal (4HNE) protein levels (panel D). A representative image of SOD2 and 4HNE protein levels and respective Ponceau images are presented in panels C and E, respectively. All data are expressed as fold-change from PRE levels (mean ± S.E.M., n = 7-10 subjects per target for mRNA data and n = 7 for 4HNE protein levels). Dashed lines represent individual response patterns. Significance from between time points comparisons using Student's t-tests are indicated within each panel.