The acute angiogenic signalling response to low load resistance exercise with blood flow restriction

Richard A. Ferguson[1], Julie E. A. Hunt[1,2], Mark P. Lewis[1], Neil R. W. Martin[1], Darren Player[1], Carolin Stangier[1,3], Conor W. Taylor[1] and Mark C. Turner[1]

[1] School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, LE11 3TU, United Kingdom.
[2] Faculty of Health and Medical Sciences, School of Biosciences & Medicine, University of Surrey, Guildford, GU2 7YW, United Kingdom.
[3] Institute of Movement and Neurosciences, German Sport University, Cologne, Germany.

Corresponding author:
Richard A. Ferguson
School of Sport, Exercise and Health Sciences
Loughborough University
Leicestershire
LE11 3TU
United Kingdom

Tel: +44 (0) 1509 226333
Fax: +44 (0) 1509 226301
E-mail: R.Ferguson@lboro.ac.uk

Abstract

This study investigated protein kinase activation and gene expression of angiogenic factors in response to low-load resistance exercise with or without blood flow restriction (BFR). In a repeated measures cross over design, six males performed 4 sets of bilateral knee extension exercise at 20% 1RM (reps per set = 30:15:15:continued to fatigue) with BFR (110 mmHg) and without (CON). Muscle biopsies were obtained from the vastus lateralis before, 2 and 4 h post-exercise. mRNA expression was determined using real-time RT-PCR. Protein phosphorylation/expression was determined using Western blot. p38MAPK phosphorylation was greater (P = 0.05) at 2 h following BFR (1.3 ± 0.8) compared to CON (0.4 ± 0.3). AMPK phosphorylation remained unchanged. PGC-1α mRNA expression increased at 2 h (5.9 ± 1.3 vs 2.1 ± 0.8; P = 0.03) and 4 h (3.2 ± 0.8 vs 1.5 ± 0.4; P = 0.03) following BFR exercise with no change in CON. PGC-1α protein expression did not change following either exercise. BFR exercise enhanced mRNA expression of VEGF at 2 h (5.2 ± 2.8 vs 1.7 ± 1.1; P = 0.02) and 4 h (6.8 ± 4.9 vs. 2.5 ± 2.7; P = 0.01) compared to CON. mRNA expression of VEGF-R2 and HIF-1α increased following BFR exercise but only eNOS were enhanced relative to CON. MMP-9 mRNA expression was not altered in response to either exercise. Acute low load resistance exercise with BFR provides a targeted angiogenic response potentially mediated through enhanced ischaemic and shear stress stimuli.

Keywords

BFR, Kaatsu, resistance training, capillaries, hypoxia

Highlights

- Gene and protein expression/activation of components of the angiogenic signalling pathway in response to low-load BFR resistance exercise compared to low-load resistance exercise alone were investigated.
- p38MAPK phosphorylation was greater following BFR. mRNA expression of PGC-1α was greater following BFR although this did not translate to a greater PGC-1α protein expression. mRNA expression of VEGF, VEGFR-2, HIF-1α and eNOS were also greater following BFR.
- These data suggest a targeted angiogenic response to BFR exercise potentially mediated through enhanced ischemic and shear stress stimuli.

Introduction

Blood flow restricted (BFR) exercise using limb occlusion cuffs is a training modality generating substantial research interest. Recent meta-analyses have demonstrated that this training stimulus is effective at stimulating increases in skeletal muscle size and strength (Slysz et al., 2016). Whilst traditional high-load (HL) resistance training may be more effective at increasing muscle strength, both HL and BFR resistance training seem to be equally effective at increasing muscle size (Lixandrão et al, 2017). Improved skeletal muscle endurance has also been observed (Kacin & Strazar, 2011) which is lilely to be due to an improved oxygen delivery/extraction by the working skeletal muscle. To this end, adaptations at various levels of the peripheral vasculature have also been observed; including the conduit artery and resistance vessels (Hunt, Galea, Tufft, Bunce, & Ferguson, 2013). Moreover, indirect assessment of skeletal muscle micro-vasculature using the capillary filtration technique has suggested an enhanced capillary growth in response to BFR exercise (Hunt et al., 2013). Although direct evidence of capillary growth has yet to be reported recent evidence suggests low load resistance exercise with BFR can enhance expression of some angiogenic genes (Larkin et al., 2012) however further analysis is required to determine the physiological/molecular mediators of this response.

Skeletal muscle capillary growth (angiogenesis) in response to exercise training is a complex process (Egginton, 2011). The targeted angiogenic response is stimulated by several physiological signals including shear stress, passive stretch of the tissue (contractile activity), low oxygen tension and metabolic activity. The signals for capillary growth differ, depending on the exercise modality, while the angiogenic response is graded according to stimulus intensity (Egginton et al., 2011). BFR exercise may stimulate angiogenesis through signals of reduced oxygen tension (Gustafsson et al., 2007) as well as an enhanced metabolic

perturbation (Krustrup, Söderlund, Relu, Ferguson, & Bangsbo, 2009; Suga et al., 2009) which mimics that of high intensity/heavy load resistance exercise. Blood flow restriction has also been shown to alter the shear stress response during (Hudlicka & Brown, 2009) and immediately after exercise (Gundermann et al., 2012), whereas the low intensity and decreased time under tension (Loenneke et al., 2012) is likely to minimise mechanical stretch.

The signalling pathways involved in angiogenesis are also complex with vascular endothelial growth factor (VEGF) being the principle growth factor and therefore an integral index of angiogenic potential (Olfert, Howlett, Wagner, & Breen, 2010). The decrease in muscle oxygen levels during low load resistance exercise with BFR (Tanimoto, Madarame, & Ishii, 2005) may stabilise hypoxia-inducible factor 1α (HIF-1α) for targeted activation of VEGF transcription. Shear stress induced production of nitric oxide (NO) via the activity of endothelial nitric oxide synthase (eNOS) has also been shown to promote VEGF efflux from skeletal muscle (Benoit, Jordan, Wagner, & Wagner, 1999) and availability at EC-receptor sites for VEGFR-2 activation (Shen et al., 1998), mediating the angiogenic effect of VEGF (Milkiewicz, Hudlicka, Brown, & Silgram, 2005). Finally, matrix metalloproteinases (MMP) increase in response to muscle stretch and mechanical loading (Høier, Rufener, Bojsen-Møller, Bangsbo, & Hellsten, 2010), and are involved in proteolysis of the capillary basement membrane, which is essential for the progression of sprouting angiogenesis (Egginton, 2011; Haas et al., 2000).

Upstream of the VEGF signalling cascade within the skeletal muscle fibres, are the key stress-activated kinase proteins AMPK and p38MAPK. p38MAPK is activated by cytosolic increases in Ca2+ (Wright, Geiger, Han, Jones, & Holloszy, 2007) as well as reactive oxygen

species (ROS) (kang, O'Moore, Dickman, & Ji, 2009) and results in an increased expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). AMPK is activated in response to an increased AMP:ATP ratio and has been demonstrated to mediate regulation of VEGF expression through PGC-1α (Leick et al., 2009). Collectively, this provides evidence that under conditions of increased energy stress and muscle activation with BFR exercise, activation of AMPK and p38MAPK, and consequent expression of PGC-1α is likely to occur.

Therefore, the purpose of the study was to examine gene and protein expression/activation of key components of the angiogenic signalling pathway in response to low load BFR resistance exercise compared to low load resistance exercise alone. It is hypothesised that BFR will activate p38MAPK and AMPK proteins. Up-regulation of PGC-1α, HIF-1α, and eNOS mRNA is expected to occur reflecting reduced oxygen tension and higher metabolic stress during BFR exercise, and greater reactive hyperaemia-induced shear stress following cuff deflation, together with an augmented increase in VEGF and VEGFR-2 mRNA expression. In contrast, mRNA expression of MMP-9 is likely to remain unchanged given the low mechanical stress during low load exercise.

Methods

Participants

Six healthy males (age; 26 ± 2 yrs, height; 184 ± 6 cm, body mass; 83 ± 11 kg, resting mean arterial pressure 93 ± 10 mmHg) volunteered to take part in the investigation. All were habitually physically active but none were specifically resistance exercise trained. The participants were fully informed of the purposes, risks and discomforts associated with the experiment before providing written, informed consent. The study conformed to current local guidelines and the Declaration of Helsinki and was approved by Loughborough University Ethics Advisory Committee.

Experimental design

In a repeated measures counter-balanced cross-over design, participants completed bilateral low load knee extension exercise on two occasions; 1) under blood flow restricted (BFR) and 2) non-restricted blood flow (CON) conditions. Muscle biopsies were obtained before, 2 and 4 hours post exercise. A minimum of 3 weeks was allowed between experimental trials and participants maintained their usual levels of physical activity throughout the study period.

Preliminary trials and familiarisation

Participants attended a preliminary visit 3 weeks prior to the experimental trial to determine maximum knee extensor strength (1RM) and conduct exercise protocol familiarisation. Bilateral maximum dynamic strength of the knee extensor muscles was assessed on a knee extension machine. The participants completed a warm up on a cycle ergometer (5 min at ~75W) before moving to the knee extension machine to perform 8 reps at 50% and 3 reps at

70% of the predicted 1RM. The load was then set to approximately 80% of the predicted 1RM. Following each successful lift the load was increased ~5% until the participant failed to lift the load through the entire range of motion (from knee joint angle of 90° through to full anatomical extension). A 4 min recovery period was allowed between each effort and 1RM achieved within 5 attempts. Participants were familiarised with the protocol of low load resistance exercise with BFR as described below. The number of repetitions performed (to fatigue) in the last set was recorded for replication in the experimental trials. This ensured a work matched design between BFR and non-BFR exercise conditions (CON), and enabled a counter-balanced order for the main experimental trial.

Experimental protocol

Participants attended the laboratory in the morning (7-8 am) after a standardised breakfast, having monitored dietary intake for 48 h (for replication in the subsequent experimental trial) and refrained from exercise for 24 h. After resting in a supine position for 20 min a muscle biopsy was attained from the vastus lateralis at a location distal to the occlusion site. This was followed by the exercise protocol which consisted of 4 sets of bilateral knee extension at 20% 1RM (1 x 30 reps, 2 x 15 reps, 1 x reps to fatigue, with 30 s recovery between sets), at a frequency of 20 contractions per minute (duty cycle of 1.5 s concentric and 1.5 s eccentric muscle actions). Range of motion was from a knee joint angle of 90° through to full anatomical extension. The number of repetitions performed in the last set was pre-determined from the preliminary visit. In the BFR trial, 13 cm wide pneumatic cuffs (SC12L segmental pressure cuff, Hokanson, WA) were wrapped around the proximal part of each thigh and inflated to a pressure of 110mmHg (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA). This occlusion pressure induces an approximate 60% decrease in resting popliteal blood flow (Hunt et al, 2016). Pressure was applied 15 s prior to

commencing exercise and maintained for the duration of the 4 sets, including the 30 s intervening rest periods (total occlusion period of 5-8 min). In the CON trial, the same exercise was performed but under normal blood flow conditions without the application of pneumatic cuffs. After completion of the exercise participants returned to the supine position and further muscle biopsies were obtained 2 and 4 hours post exercise.

Muscle biopsy sampling and analysis

Muscle biopsies were obtained from the vastus lateralis muscle under local anesthesia (1% lidocaine) using the micro-biopsy technique (Acecut 11G Biopsy Needle, TSK). Two passes were taken at each time point (average tissue mass: 29.4 ± 8.3 mg). One sample was immediately placed into tubes containing 500µl TRIzol reagent (Sigma-Aldrich 93289) and frozen in liquid nitrogen before storage at -80°C. The other sample was immediately snap frozen in liquid nitrogen before storage at -80°C. Biopsies were alternated between legs and sites were separated by at least 2 cm. The resting biopsy sample was counterbalanced to dominant and non-dominant legs.

RNA isolation. Muscle samples snap frozen in 500µl TRIzol reagent were thawed and gently homogenised over ice, using a handheld TissueRuptor probe (QIAGEN). The solution was separated into an aqueous phase and an organic phase following the addition of chloroform and centrifugation. RNA was isolated from the aqueous phase following precipitation with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, dried and re-suspended in 50 µl of RNA storage solution (Ambion® AM7001). Sample RNA concentration (631 ± 306 ng/µl) and purity (260/280: 2.0 ± 0.2, 260/230: 1.6 ± 0.3) was confirmed using spectrophotometry (Nanodrop).

Real-time qualitative RT-PCR. Muscle mRNA expression was determined using real-time PCR with primer sequences designed by SIGMA (Table 1). RNA was quantified

fluorometrically using QuantiFast SYBR Green one step RT-PCR Kit (QIAGEN), performed in duplicate (70 ng of RNA in a total reaction volume of 20 µl). Relative quantification was determined using the Mx3000P/Mx3005P QPCR cycler (Stratagene, Agilent Tehnologies) with universal cycling conditions (one cycle at 50^{o}C for 10 min [RT] and 95^{o}C for 5 min, 40 cycles of denaturation at 95^{o}C for 10 s and annealing/extension at 60^{o}C for 30 s). Fluorescence was measured after each of the repeated cycles. On completion of PCR, all PCR products formed were melted to attain a melting curve profile, which confirmed reaction specificity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene as it remained stable across time for both trials. Results were expressed using the comparative cycle threshold (Ct) method. Post exercise values were reported as a fold difference relative to the expression of the pre-exercise values. Relative fold changes were determined using the $2^{-\Delta\Delta Ct}$ method using the equation: Fold = $2^{\text{(Ct target gene- Ct housekeeping gene) time x – (Ct mean target gene-Ct mean housekeeping gene) baseline.}}$

Immunoblotting. Muscle samples were homogenised in cold lysis buffer containing PBS-0.2%, Triton X-100 and protease and phosphatase inhibitor cocktail (Fisher Scientific). Samples were blitzed using a tissue lyser (Qiagen) twice for 2 min at 20Hz and centrifuged at 12,000 g for 10 minutes to pellet insoluble material. The supernatant was removed to a fresh Eppendorf tube and protein concentrations were determined by Pierce 660 nM protein assay according to the manufacturer's instructions (Fisher Scientific). Samples were mixed with dH_2O, 4X LDS sample buffer (Invitrogen) and 0.1% β-mercaptoethanol (Sigma) to a concentration of 2.5 µg·µL and boiled for 5 min at 95°C. A protein concentration of 25 µg was loaded on to 4-12% Criterion Bis-Tris gels (Bio-Rad) and separated by electrophoresis. Proteins were transferred onto PVDF membrane (Bio-Rad) and washed for 5 min in Tris buffered saline with tween (TBST) before being blocked in 5% blotting grade milk (Bio-Rad) for 1 h at 4°C. Membranes were washed 3 x 5 minutes in TBST before being incubated

overnight with appropriate primary antibody at 4°C. Antibodies, AMPK (#2532), $AMPK^{Thr172}$ (#4188), p38MAPK (#9212), $p38MAPK^{Thr180/Tyr182}$ (#9215), GAPDH (#2118) (Cell Signalling, New England Biolabs), PGC-1α (#ab191838) (Abcam). The following morning, membranes were washed for 3 × 5 min in TBST, and subsequently incubated with anti-species horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 hr at room temperature. After 3 × 5 min washes in TBST, membranes were incubated with chemiluminescence (ClarityMax, Bio-Rad) for 5 min prior to exposure. Membranes were visualised using image analysis (ChemiDoc™ XRS+, BioRad), and band densities determined using image analysis software (Quality One 1-D analysis software v 4.6.8, Bio-Rad). All raw densitometry data were used for statistical analysis to compare within-subject responses to both conditions (BFR, CON) and presented as such for the signaling proteins (p38MAPK and AMPK). For graphical purposes results for PGC-1α protein expression was normalized to 1 for each subject's pre-exercise value in both conditions (hence no error bars are shown for this time point on Figs. 2B). Accordingly, values at 2 h and 4 h post-exercise are expressed as fold change relative to pre-exercise values.

Statistics

A Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. When the data was normally distributed (in the case of AMPK and p38MAPK phsophorlylation, PGC-1α protein and VEGF mRNA) a parametric two-way (condition x time) ANOVA with repeated measures was carried out. When the data was not normally distributed (in the case of VEGFR-2, PGC-1α, HIF-1α, eNOS and MMP-9 mRNA) a non-parametric Friedman's test was carried out. Targeted pairwise comparisons were used to assess differences within trials with respect to pre-exercise and between trials at the same time point using paired t-tests and Wilcoxon signed

rank tests for parametric and non-parametric data, respectively. Effect sizes were calculated by partial eta squared (partial η2) for the two-way ANOVA with repeated measures and Cohen's d (d) for the subsequent pairwise comparisons following the paired t-tests and Kendalls W for the pairwise comparisons following the Wilcoxon tests. Differences were considered significant at $P \leq 0.05$. All data are presented as mean ± SD, unless otherwise stated.

Results

Bilateral knee extension 1RM was 141 ± 21 kg. The exercise protocol was performed at 20% of 1RM, equating to 28 ± 4 kg. Following the first three sets of exercise which were performed for 30, 15 and 15 reps, respectively, the number of repetitions completed in the final set to fatigue was 37 ± 28, and varied greatly between individuals (range 4 - 81 reps). The total number of repetitions performed in the exercise bouts (BFR & CON) was therefore 97 ± 28 and amounted to 397 ± 85 s under occlusion.

There was a significant effect for time (P = 0.05, partial η^2 = 0.57, observed power = 0.54) and condition (P = 0.04, partial η^2 = 0.62, observed power = 0.62) for p38MAPK phosphorylation (Fig. 1); the condition x time interaction was close to significance (P = 0.08, partial η^2 = 0.49, observed power = 0.43). Post-hoc analysis revealed a strong trend (and large effect size) to increase p38MAPK phosphorylation in the BFR group (P = 0.06, d = 1.49) and there was a difference (with a large effect size) between conditions at 2 h (P = 0.05, d = 1.42). There were no main effects for time (P = 0.31, partial η^2 = 0.20), condition (P = 0.45, partial η^2 = 0.12) or condition x time interaction (P = 0.43, partial η^2 = 0.13) for AMPK phosphorylation (Fig. 1).

A significant Friedman's test (P = 0.01) and subsequent post-hoc tests revealed PGC-1α mRNA expression (Fig. 2) increased 2 h (P = 0.03, W = 1.00) and 4 h (P = 0.03, W = 1.00) after BFR exercise, with no change in CON. PGC-1α mRNA expression was greater in BFR compared to CON at 4 h (P = 0.05, W = 0.44) but not 2 h (P = 0.07, W = 0.44). There were no main effects for time (P = 0.20, partial η^2 = 0.27), condition (P = 0.22, partial η^2 = 0.28)

or condition x time interaction (P = 0.15, partial η^2 = 0.32) for PGC-1α protein expression (Fig. 2).

There was a significant effect for time (P = 0.03, partial η2 = 0.51, observed power = 0.70), condition (P = 0.01, partial η2 = 0.78, observed power = 0.91) and a condition x time interaction (P = 0.01, partial η2 = 0.62, observed power = 0.89) for VEGF mRNA expression (Fig. 3). Post-hoc analysis revealed that VEGF mRNA expression increased 2 h (P = 0.02, d = 1.99) and 4 h (P = 0.04, cohen d = 1.61) after BFR exercise with no change observed in CON. VEGF mRNA was greater in BFR compared to the CON group at 2 h (P = 0.02, d = 1.61) and 4 h (P = 0.01, d = 1.08).

There was a trend for VEGFR-2 mRNA expression to change after BFR exercise (Friedman's test P = 0.07). A subsequent a priori comparison revealed VEGFR-2 mRNA expression (Fig. 3) increased at 4 h (P = 0.05, ES = 0.44) after BFR exercise with no change in CON. However, VEGFR-2 mRNA did not differ between conditions at 4 h (P = 0.08, ES = 0.44).

A significant Friedman's test (P = 0.03) and subsequent post-hoc tests revealed eNOS mRNA expression (Fig. 3) increased 2 h (P = 0.03, W = 0.11) but not at 4 h (P = 0.25) after BFR exercise, with no change in CON. However, a difference between conditions was only evident at 4 h post exercise (P = 0.05, W = 0.44).

A significant Friedman's test (P = 0.05) and subsequent post-hoc tests revealed HIF-1α mRNA expression (Fig. 3) was increased 2 h (P = 0.03, W = 1.00) after BFR exercise. However, no further differences were detected. Large between-subject variability in the HIF-1α mRNA response at 4 h after BFR exercise precluded significance (P = 0.12).

MMP-9 mRNA expression (Fig. 3) did not change in response to either CON or BFR exercise (Friedman's test $P = 0.34$).

Discussion

This study has demonstrated a greater p38MAPK phosphorylation following low load resistance exercise with BFR, compared with low-load resistance exercise alone. BFR also potentiates the mRNA expression of PGC-1α although this did not translate to a greater PGC-1α protein expression. mRNA expression of VEGF, VEGFR-2, HIF-1α and eNOS were upregulated following low load resistance exercise with BFR. These data suggest a targeted angiogenic response to BFR exercise potentially mediated through enhanced ischemic and shear stress stimuli.

The greater phosphorylation of p38MAPK is perhaps not surprising given it is considered to be a stress-activated kinase and has been repeatedly shown to be activated in response to various exercise modalities including resistance training (Camera, Hawley, & Coffey, 2015). p38MAPK lies downstream of the calcium/calmodulin-dependent protein kinase (CAMK) which is activated by cytosolic increases in Ca^{2+}, that ultimately leads to increased PGC-1α expression (Wright et al., 2007). The key physiological stimulus causing the release of Ca^{2+} from the sarcoplasmic reticulum is depolarisation of the muscle surface membrane in the form of an action potential (Endo, 1977). Thus, an increase in motor unit activity (i.e. EMG activity) which has been reported during BFR resistance exercise (Fatela, Reis, Mendonca, Avela & Mil-Homens, 2016) likely explains the greater phosphorylation of p38MAPK. Moreover, reactive oxygen species (ROS) generated in response to ischemia-reperfusion and muscle contraction can also activate MAPK and PGC-1α signalling pathways (Kang et al., 2009) and may contribute to the phosphorylation of p38MAPK following BFR resistance exercise. However, ROS production during BFR resistance exercise may be minor given that levels of oxidative stress markers are unaltered post exercise (Nielsen et al., 2017). It is,

however, surprising that AMPK was not similarly activated given the additional metabolic stimulus BFR has been purported to provide (Krustrup et al., 2009; Suga et al., 2009). This may be reflected by the specific protocol used in the present study (1 x 30 reps, 2 x 15 reps, 1 x reps to fatigue, with 30 s recovery between sets) which may not have been as metabolically challenging as expected.

This study is the first to demonstrate that BFR potentiates the expression of PGC-1α mRNA in response to low load knee extension exercise. The magnitude and time-course of the PGC-1α mRNA response in the present study is comparable with others (Mathai, Bonen, Benton, Robinson, & Graham, 2008) and further reflects the potential for BFR exercise to promote angiogeneisis and mitochondrial biogenesis given the role of PGC-1α in both adaptive processes (Chinsomboon et al., 2009). However, as has been demonstrated previously during other exercise modalities there was no concomitant changes in PGC-1α protein, which reflects the observation that translation of PGC-1α protein only occurs several days after exercise (Perry et al., 2010).

In agreement with previous observations (Larkin et al., 2012) the present study demonstrates that BFR can increase VEGF transcription in response to low load bilateral knee extension exercise. The magnitude of this response (5.2- and 6.7-fold increase in VEGF mRNA at 2 and 4 h post BFR exercise) exceeds that elicited by heavy load resistance exercise (Gavin, Drew, Kubik, Pofahl, & Hickner, 2007) but is similar to that obtained after longer duration (~45 min) BFR protocols (Ameln et al., 2005; Gustafsson et al., 2007). A similar VEGF response despite shorter exercise duration may be attributable to the greater reduction of leg blood flow elicited by direct occlusion cuff application (60% reduced popliteal artery flow; Hunt et

al. 2016) compared to pressure chamber induced BFR (15-20% reduced femoral artery flow; Sundberg & Kaijser 1992).

VEGFR-2, the major mediator of VEGF induced angiogenesis (Milkiewicz et al., 2005), increased 4.6-fold following low load BFR resistance exercise, exceeding the response observed previously to heavy load resistance exercise (Gavin et al., 2007). However, this was largely mediated by one participants response, and in contrast to previous investigations (Larkin et al., 2012) VEGFR-2 transcriptional response did not differ between exercise conditions although a near significant trend was observed ($p=0.08$, 4 h post exercise). An enhanced secretion of VEGF by muscle fibres during BFR exercise (Takano et al., 2005) may increase VEGF binding to VEGFR-2 and explain the increase in VEGFR-2 gene transcription (Shen et al., 1998). Alternatively the up-regulation of VEGFR-2 mRNA may result from the increased number of endothelial cells given the enhanced proliferation of EC (Gustafsson et al., 2007) after BFR exercise.

In the present study we also examined mRNA expression of HIF-1α, eNOS and MMP-9 as recognised markers of hypoxic, shear and mechanical stress mediated angiogenic signalling, respectively. Although HIF-1α mRNA increased following BFR exercise the response did not differ to the control condition, which has been observed before (Ameln et al., 2005; Drummond et al., 2008) suggesting regulation by HIF-1α may not account for the augmented VEGF expression in response to BFR resistance exercise. Alternatively VEGF may be regulated in a HIF-1α independent manner by PGC-1α (Arany et al., 2008). The pro-angiogenic actions of shear stress facilitate angiogenesis (Adair & Montani, 2010). eNOS mRNA was elevated after BFR exercise and peak expression occurred prior to that of VEGF and VEGFR-2, consistent with the hypothesis that shear stress induced NO release functions

as an upstream regulator of the VEGF signalling pathway (Milkiewicz et al., 2005). The enhanced eNOS expression following BFR exercise may be a consequence of the shear stimulus elicited by reactive hyperaemia following cuff deflation (Gundermann et al., 2012). However, increased circumferential strain (stretch) as a result of raised capillary pressure with proximal cuff inflation (venous occlusion) (Price & Skalak, 1994), and mechanical compression of the vessels with skeletal muscle contraction (Chen et al., 2002) may also explain findings. In contrast, there was no change in eNOS mRNA in the control trial, which may reflect the modest hyperemia associated with short duration low intensity resistance exercise and elicited a mechanical stimulus (shear stress, circumferential strain and/or compression) that was below the threshold required to stimulate eNOS transcription (Balligand, Feron, & Dessy, 2009). Neither bout of low load knee extension exercise elicited a change in MMP-9 mRNA which probably reflects the reduced time under tension (Loenneke et al., 2012) and minimised dynamic stretch of the tissue that is typical of low-load exercise.

The present study is not without limitations. As with similar reports (Larkin et al., 2012), despite having a low sample size we were able to detect significant differences with adaquate statistical power. Where ‘trends’ were reported the calculated effect sizes could be considered to be large in magnitude, further supporting our interpretations. We also appreciate that a sampling time of 2 h post-exercise is not the optimal time to assess phosphorylation status of AMPK and p38MAPK, especially since activation of these proteins usually occur in the immediate period (< 1 h) following exercise (e.g. Camera et al., 2015). We concede that the primary purpose of the study was to examine the gene expression responses hence the 2 and 4 h muscle sampling time points and we took an opportunistic approach to the analysis of AMPK and p38MAPK. Nevertheless, this data does provide an insight into the initial acute

signals for the adaptive response following low load resistance exercise with BFR which requires further investigation. We also appreciate that given the acute nature of this experiment, there are limitations in predicting chronic adaptations to BFR training. However, previous studies have demonstrated that the acute angiogenic gene response does inform the magnitude of capillary growth with training in that increases in acute exercise-induced VEGF, VEGFR-2 and eNOS mRNA expression are associated with a significant capillarisation response after 4 weeks of training (e.g. Høier et al. 2012).

In conclusion, acute low load resistance exercise with BFR provides a targeted angiogenic response potentially mediated through enhanced ischaemic and shear stress stimuli. A more detailed insight into the angiogenic cascade is required with measurement of further pro- and anti-angiogenic factors to determine the modulation of angiogenesis and direct measures of capillary growth in response to BFR exercise training are now required.

Acknowledgements We thank the participants for their effort and time commitment.

Conflict of interest The authors have no conflict of interest.

References

Adair, T. H., & Montani, J.-P. (2010). Angiogenesis. *Colloquium Series on Integrated Systems Physiology: From Molecule to Function*, 2(1), 1–84.

Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., & Makino, Y. (2005). Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, 19(8), 1009–1011.

Arany, Z., Foo, S-Y, Ma, Y., Ruas, J. L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S. M., Baek, K. H., Rosenzweig, A., & Spiegelman, B. M. (2008). HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1α. *Nature*, *451*(7181), 1008–1012.

Balligand, J., Feron, O., & Dessy, C. (2009). eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiological Reviews. 89(2), 481-534.

Benoit, H., Jordan, M., Wagner, H., & Wagner, P. D. (1999). Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. *Journal of Applied Physiology*, 86(5), 1513–1518.

Camera, D. M., Hawley, J. A., & Coffey, V. G. (2015). Resistance exercise with low glycogen increases p53 phosphorylation and PGC-1α mRNA in skeletal muscle. *European Journal of Applied Physiology*, 115(6), 1185–1194.

Chen, L.-E., Liu, K., Qi, W.-N., Joneschild, E., Tan, X., Seaber, A. V., Stamler, J. S., & Urbaniak, J. R. (2002). Role of nitric oxide in vasodilation in upstream muscle during intermittent pneumatic compression. *Journal of Applied Physiology*, 92(2), 559–566.

Chinsomboon, J., Ruas, J., Gupta, R. K., Thom, R., Shoag, J., Rowe, G. C., Sawada, N., Raghuram, S., & Arany, Z. (2009). The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America*, 106(50), 21401–21406.

Drummond, M. J., Fujita, S., Abe, T., Takashi, A., Dreyer, H. C., Volpi, E., & Rasmussen, B. B. (2008). Human muscle gene expression following resistance exercise and blood flow restriction. *Medicine and Science in Sports and Exercise*, 40(4), 691–698.

Egginton, S. (2011). Physiological factors influencing capillary growth. *Acta Physiologica*, 202(3), 225–239.

Egginton, S., Badr, I., Williams, J., Hauton, D., Baan, G. C., & Jaspers, R. T. (2011). Physiological angiogenesis is a graded, not threshold, response. *Journal of Physiology*, 589(1), 195-206.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Reviews*, 57(1), 71-108.

Fatela, P., Reis, J. F., Mendonca, G. V., Avela J. & Mil-Homens, P. (2016). Acute effects of exercise under different levels of blood-flow restriction on muscle activation and fatigue. *European Journal of Applied Physiology*, 116(5), 985-995.

Gavin, T. P., Drew, J. L., Kubik, C. J., Pofahl, W. E., & Hickner, R. C. (2007). Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiologica*, 191(2), 139–146.

Gundermann, D. M., Fry, C. S., Dickinson, J. M., Walker, D. K., Timmerman, K. L.,

Drummond, M. J., Volpi, E., & Rasmussen, B. B. (2012). Reactive hyperemia is not responsible for stimulating muscle protein synthesis following blood flow restriction exercise. *Journal of Applied Physiology*, 112(9), 1520–1528.

Gustafsson, T., Rundqvist, H., Norrbom, J., Rullman, E., Jansson, E., & Sundberg, C. J. (2007). The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle. *Journal of Applied Physiology*, 103(3), 1012–1020.

Haas, T. L., Milkiewicz, M., Davis, S. J., Zhou, A. L., Egginton, S., Brown, M. D., Madri, J. A., Hudlicka, O. (2000). Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. *American Journal of Physiology. Heart and Circulatory Physiology*, 279(4), H1540-H1547.

Høier, B., Nordsborg, N., Andersen, S., Jensen, L., Nybo, L., Bangsbo, J., & Hellsten, Y. (2012). Pro- and anti-angiogenic factors in human skeletal muscle in response to acute exercise and training. *Journal of Physiology*, 590(3), 595–606.

Høier, B., Rufener, N., Bojsen-Møller, J., Bangsbo, J., & Hellsten, Y. (2010). The effect of passive movement training on angiogenic factors and capillary growth in human skeletal muscle. *Journal of Physiology*, 588(19), 3833–3845.

Hudlicka, O., & Brown, M. D. (2009). Adaptation of skeletal muscle microvasculature to increased or decreased blood flow: role of shear stress, nitric oxide and vascular endothelial growth factor. *Journal of Vascular Research*, 46(5), 504–512.

Hunt, J. E. A., Stodart, C., & Ferguson, R. A. (2016). The influence of participant characteristics on the relationship between cuff pressure and level of blood flow restriction. *European Journal of Applied Physiology*, 116(7), 1421–1432.

Hunt, J. E. A., Galea, D., Tufft, G., Bunce, D., & Ferguson, R. A. (2013). Time course of regional vascular adaptations to low load resistance training with blood flow restriction. *Journal of Applied Physiology*, 115(3), 403–411.

Kacin, A., & Strazar, K. (2011). Frequent low-load ischemic resistance exercise to failure enhances muscle oxygen delivery and endurance capacity. *Scandinavian Journal of Medicine & Science in Sports*, 21(6), e231-e241.

Kang, C., O'Moore, K. M., Dickman, J. R., & Ji, L. L. (2009). Exercise activation of muscle peroxisome proliferator-activated receptor-γ coactivator-1α signaling is redox sensitive. *Free Radical Biology and Medicine*, 47(10), 1394–1400.

Krustrup, P., Söderlund, K., Relu, M. U., Ferguson, R. A, & Bangsbo, J. (2009). Heterogeneous recruitment of quadriceps muscle portions and fibre types during moderate intensity knee-extensor exercise: effect of thigh occlusion. *Scandinavian Journal of Medicine & Science in Sports*, 19(4), 576–584.

Larkin, K. A, Macneil, R. G., Dirain, M., Sandesara, B., Manini, T. M., & Buford, T. W. (2012). Blood flow restriction enhances post-resistance exercise angiogenic gene expression. *Medicine and Science in Sports and Exercise*, 44(11), 2077–2083.

Leick, L., Hellsten, Y., Fentz, J., Lyngby, S. S., Wojtaszewski, J. F. P., Hidalgo, J., & Pilegaard, H. (2009). PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. *American Journal of Physiology. Endocrinology and Metabolism*, 297(1), E92-E103.

Lixandrão, M. E., Ugrinowitsch, C., Berton, R., Vechin, F. C., Conceição, M. S., Damas, F., Libardi, C. A., & Roschel, H. (2017). Magnitude of muscle strength and mass adaptations between high-load resistance training versus low-load resistance training

associated with blood-flow restriction: a systematic review and meta-analysis. *Sports Medicine*, doi: 10.1007/s40279-017-0795-y (Epub ahead of print).

Loenneke, J. P., Wilson, J. M., Balapur, A., Thrower, A. D., Barnes, J. T., & Pujol, T. J. (2012). Time under tension decreased with blood flow-restricted exercise. *Clinical Physiology and Functional Imaging*, 32(4), 268–273.

Mathai, A. S., Bonen, A., Benton, C. R., Robinson, D. L., & Graham, T. E. (2008). Rapid exercise-induced changes in PGC-1alpha mRNA and protein in human skeletal muscle. *Journal of Applied Physiology*, 105(4), 1098–1105.

Milkiewicz, M., Hudlicka, O., Brown, M. D., & Silgram, H. (2005). Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle. *American Journal of Physiology. Heart and Circulatory Physiology*, 289(1), H336-H343.

Nielsen, J. L., Aagaard, P., Prokhorova, T. A., Nygaard, T., Bech, R. D., Suetta, C., & Frandsen, U. (2017). Blood flow restricted training leads to myocellular macrophage infiltration and upregulation of heat shock proteins , but no apparent muscle damage. *Journal of Physiology*, 595(14), 4857-4873.

Olfert, I. M., Howlett, R. A, Wagner, P. D., & Breen, E. C. (2010). Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 299(4), R1059-R1067.

Perry, C. G. R., Lally, J., Holloway, G. P., Heigenhauser, G. J. F., Bonen, A., & Spriet, L. L. (2010). Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *Journal of Physiology*, 588(23), 4795–4810.

Price, R. J., & Skalak, T. C. (1994). Circumferential wall stress as a mechanism for arteriolar rarefaction and proliferation in a network model. *Microvascular Research*, 47(2), 188–202.

Shen, B. Q., Lee, D. Y., Gerber, H. P., Keyt, B. A., Ferrara, N., & Zioncheck, T. F. (1998). Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. *Journal of Biological Chemistry*, 273(45), 29979–29985.

Suga, T., Okita, K., Morita, N., Yokota, T., Hirabayashi, K., Horiuchi, M., Takada, S., Takahashi, T., Omokawa, M., Kinugawa, S., & Tsutsui, H. (2009). Intramuscular metabolism during low-intensity resistance exercise with blood flow restriction. *Journal of Applied Physiology*, 106(4), 1119–1124.

Slysz, J., Stultz, J., & Burr, J. F. (2016). The efficacy of blood flow restricted exercise: A systematic review & meta-analysis. Journ*al of Science and Medicine in Sport*, 19(8), 669-675.

Sundberg, C. J., & Kaijser, L. (1992). Effects of graded restriction of perfusion on circulation and metabolism in the working leg; quantification of a human ischaemia-model. *Acta Physiologica Scandinavica*, 146(1), 1–9.

Takano, H., Morita, T., Iida, H., Asada, K., Kato, M., Uno, K., Hirose, K., Matsumoto, A., Takenaka, K., Hirata, Y., Eto, F., Nagai, R., Sato, Y., & Nakajima, T. (2005). Hemodynamic and hormonal responses to a short-term low-intensity resistance exercise with the reduction of muscle blood flow. *European Journal of Applied Physiology*, 95(1), 65–73.

Tanimoto, M., Madarame, H., & Ishii, N. (2005). Muscle oxygenation and plasma growth

hormone concentration during and after resistance exercise: Comparison between “KAATSU” and other types of regimen. *International Journal of KAATSU Training Research*, 1(2), 51–56.

Wright, D. C., Geiger, P. C., Han, D. H., Jones, T. E., & Holloszy, J. O. (2007). Calcium induces increases in peroxisome proliferator-activated receptor γ coactivator-1α and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *Journal of Biological Chemistry*, 282(26), 18793–18799.

Table legend

Table 1. DNA oligonucleotide primers used for real time PCR

Figure legends

Fig. 1 Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ and phosphorylation of AMPK^{Thr172} before and 2 h post blood flow restricted (BFR, filled bars) and non-restricted (CON, open bars) exercise. Changes in phosphorylation are expressed relative to total p38MAPK and total AMPK protein, respectively. Bars represent the mean with individual values (n = 6). † Significantly different to CON trial ($P<0.05$).

Fig. 2 Skeletal muscle mRNA and protein expression of proliferator-activated receptor-γ coactivator 1α (PGC-1α) before, 2 h and 4 h post blood flow restricted (BFR, filled bars) and non-restricted (CON, open bars) exercise. Changes in mRNA and protein content are expressed as a fold change from baseline. Bars represent the mean with individual values (n = 6). * Significantly different to pre-exercise, † Significantly different to CON trial ($P<0.05$).

Fig. 3 Skeletal muscle mRNA expression of vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR-2), hypoxia inducible factor 1α (HIF-1α) endothelial nitric oxide synthase (eNOS) and matrix metalloproteinase 9 (MMP-9) before, 2 h and 4 h post blood flow restricted (BFR, filled bars) and non-restricted (CON, open bars) exercise. Changes in mRNA content are expressed as a fold change from baseline. Bars represent the mean with individual values (n=6). * Significantly different to pre-exercise, † Significantly different to CON trial ($P<0.05$).

Figure 1

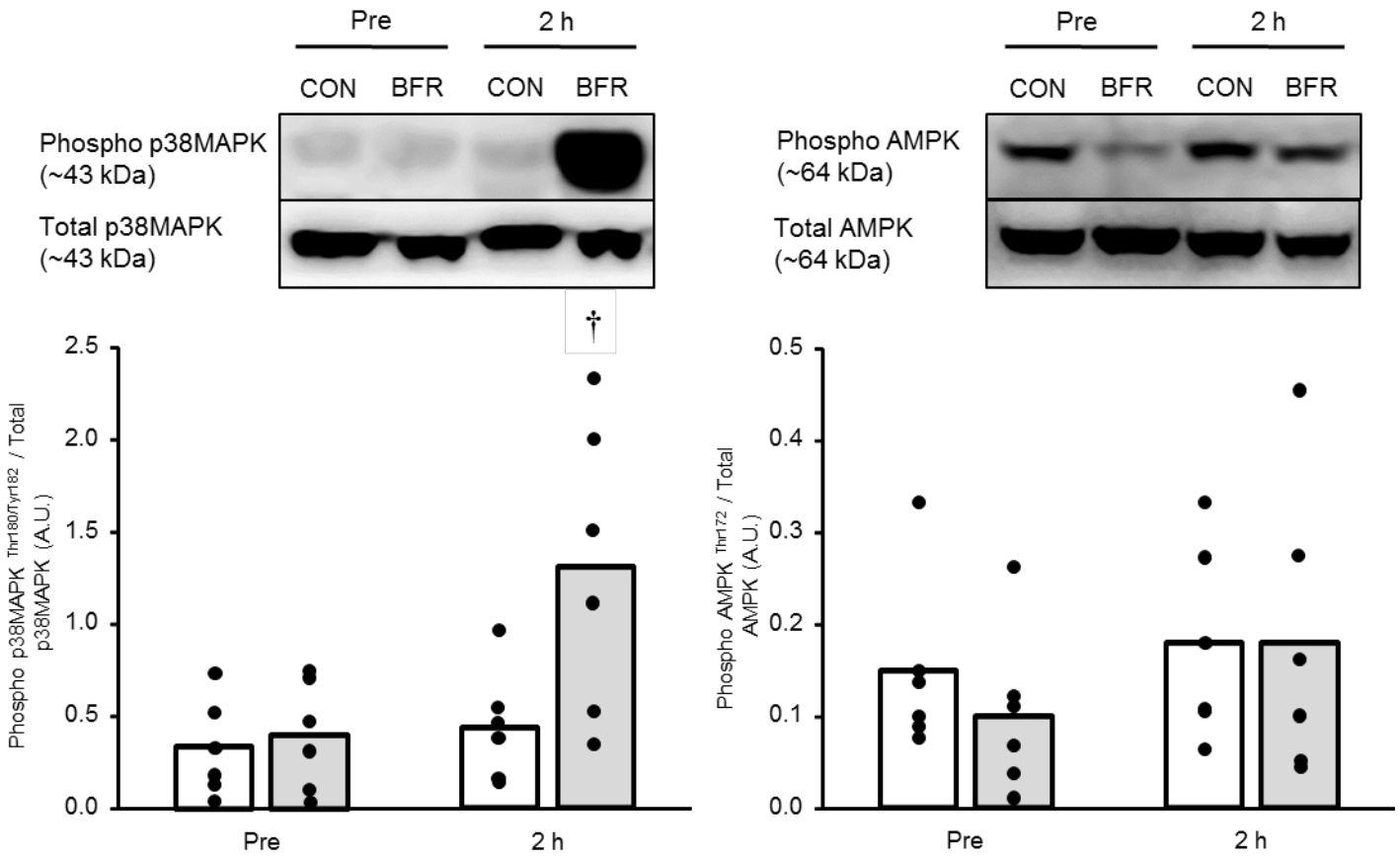
Pre
2 h
CON
BFR
CON
BFR
Phospho p38MAPK
(~43 kDa)
Total p38MAPK
(~43 kDa)
†
Phospho p38MAPK Thr180/Tyr182 / Total p38MAPK (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
Pre
2 h
Pre
2 h
CON
BFR
CON
BFR
Phospho AMPK
(~64 kDa)
Total AMPK
(~64 kDa)
Phospho AMPK Thr172 / Total AMPK (A.U.)
0.5
0.4
0.3
0.2
0.1
0.0
Pre
2 h

Figure 2

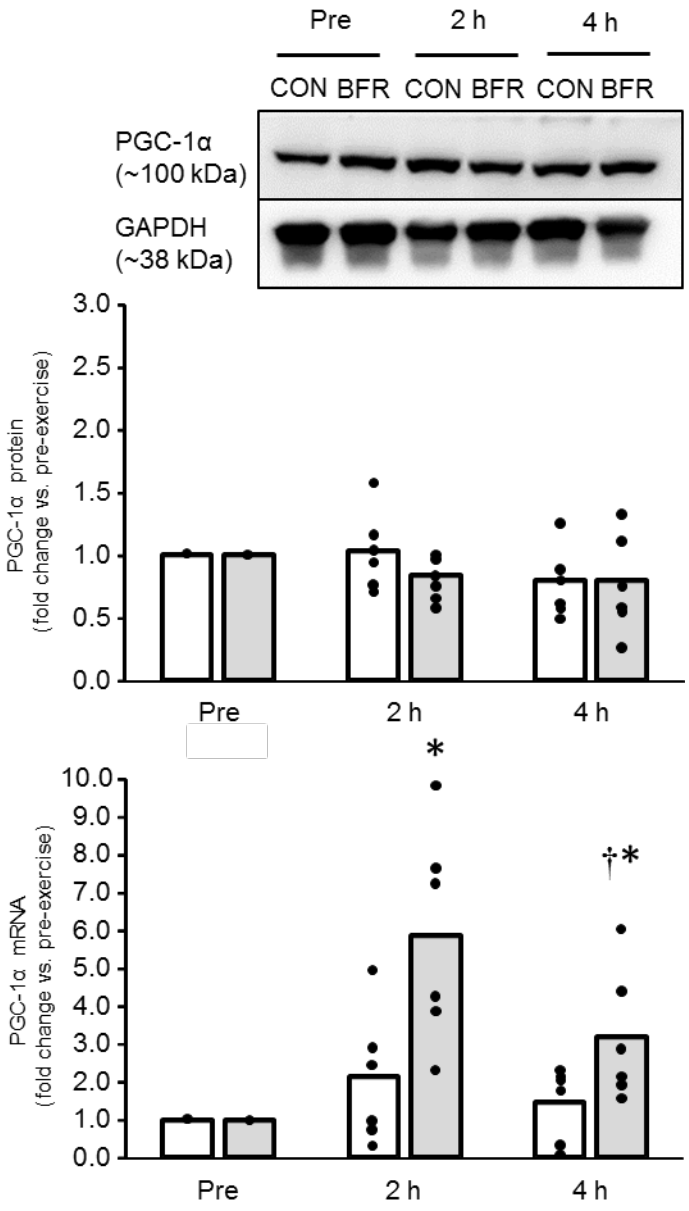
Pre
2 h
4 h
CON BFR CON BFR CON BFR
PGC-1α
(~100 kDa)
GAPDH
(~38 kDa)
PGC-1α protein
(fold change vs. pre-exercise)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
2 h
4 h
PGC-1α mRNA
(fold change vs. pre-exercise)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
†*
Pre
2 h
4 h

Figure 3

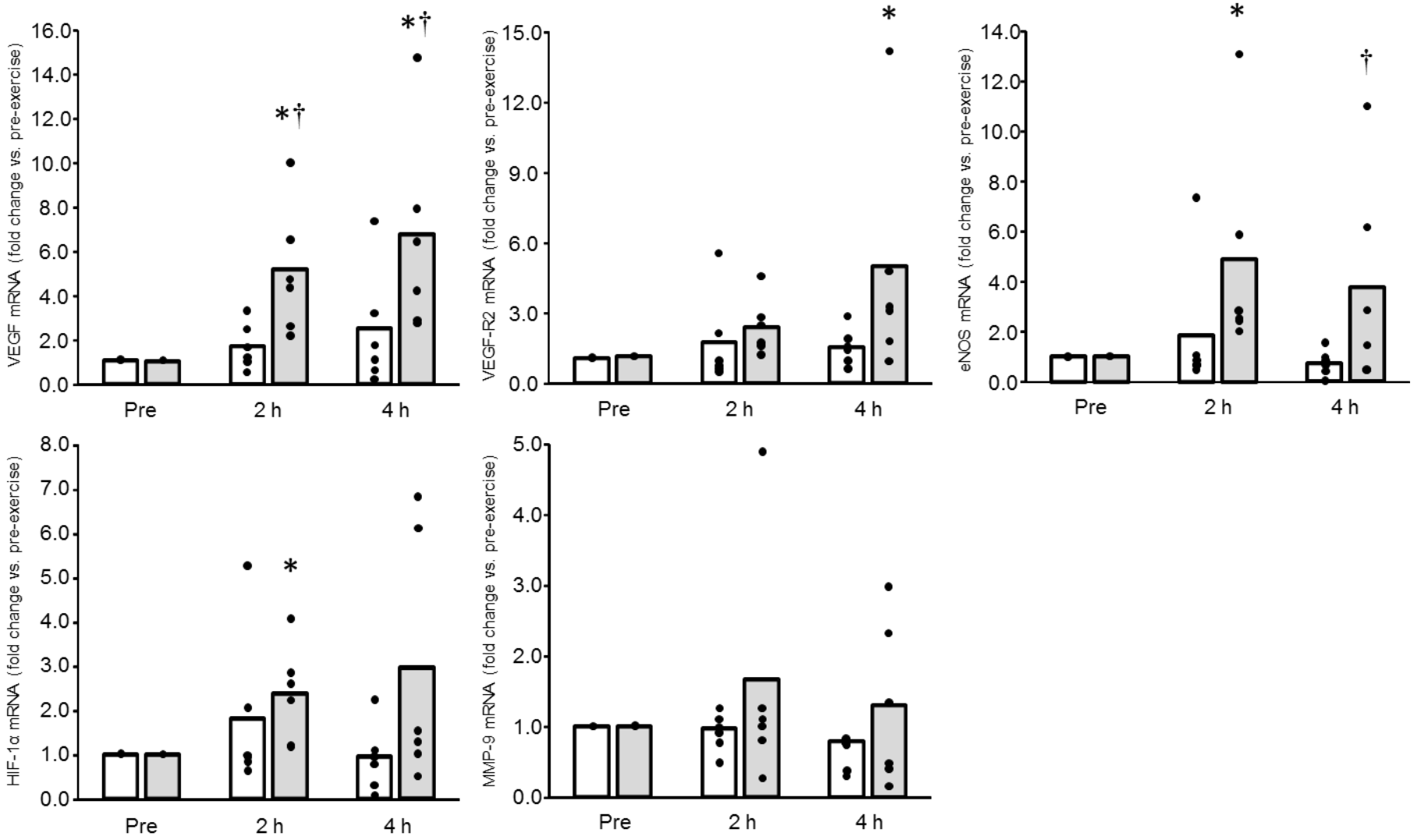
VEGF mRNA (fold change vs. pre-exercise)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*†
*†
Pre
2 h
4 h
VEGF-R2 mRNA (fold change vs. pre-exercise)
15.0
12.0
9.0
6.0
3.0
0.0
*
Pre
2 h
4 h
eNOS mRNA (fold change vs. pre-exercise)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
†
Pre
2 h
4 h
HIF-1α mRNA (fold change vs. pre-exercise)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
Pre
2 h
4 h
MMP-9 mRNA (fold change vs. pre-exercise)
5.0
4.0
3.0
2.0
1.0
0.0
Pre
2 h
4 h

Table 1.

Target mRNA	Sense Sequence (5’-3’)	Antisense Sequence (5’-3’)
VEGF	ATGAACTTCACCACTTCGT	CTGCTCTACCTCCACCAT
VEGFR-2	TTACTTCTGGTTCTTCTAACG	AGCAGGAATCAGTCAGTAT
PGC-1α	ACTCTCGCTTCTCATACTC	CCTCTTCAAGATCCTGCTA
HIF-1α	AATCTGTGTCCTGAGTAGAA	TCACCTGAGCCTAATAGTC
eNOS	TGTGAAGGCTGTAGGTTAT	CAAGTTGGAATCTCGTGAA
MMP-9	GGCACCTCTATGGTCCTC	AGTAGTGGCCGTAGAAGG
GAPDH	GGTGGAATCATATTGGAACAT	GAGTCAACGGATTTGGTC