Acute and chronic effect of sprint interval training combined with post-exercise blood flow restriction in trained individuals

Conor W Taylor[1,2], Stephen A Ingham[2], Richard A Ferguson[1]

[1] School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, LE11 3TU, United Kingdom

[2] English Institute of Sport, Performance Centre, Loughborough University, Leicestershire, LE11 3TU, United Kingdom

Corresponding author:

Richard A. Ferguson

School of Sport, Exercise and Health Sciences

Loughborough University

LE11 3TU

United Kingdom

Tel: +44 (0) 1509 226333

Fax: +44 (0) 1509 226301

Email: R.A.Ferguson@lboro.ac.uk

Running title: Blood-flow restricted sprint interval training

Keywords: HIT; BFR; maximal oxygen uptake, angiogenesis

Total words: 6,888 (including title page, abstract, legends and references)

Total number of references: 46

Subject area: Human, environmental and exercise physiology

This is an Accepted Article that has been peer-reviewed and approved for publication in the Experimental Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an Accepted Article; doi: 10.1113/EP085293 .

New Findings

What is the central question of this study?

Does the combination of sprint interval training with post-exercise blood flow restriction enhance maximal aerobic physiology and performance in trained individuals?

What is the main finding and its importance?

We demonstrate the potency of combining blood flow restriction with sprint interval training in increasing $\dot{V}O_{2max}$ in trained individuals; however this did not translate to an enhanced exercise performance**.** We also show that BFR combined with SIT enhanced post-exercise HIF-1α mRNA expression, suggesting the possibility for greater hypoxia mediated adaptations, such as enhanced capillary growth, with this intervention.

Abstract

This investigation assessed the efficacy of sprint interval training (SIT) combined with post-exercise blood flow restriction as a novel approach to enhance maximal aerobic physiology and performance. In Study 1, a between groups design determined whether 4 weeks (2 d/wk) of SIT (repeated 30 s maximal sprint cycling) combined with post-exercise blood flow restriction (BFR) enhanced maximal oxygen uptake ($\dot{V}O_{2max}$) and 15km cycling time trial performance (15km-TT) compared to SIT alone (CON) in trained individuals. $\dot{V}O_{2max}$ increased after BFR by 4.5% (P = 0.01) but was unchanged after CON. There was no difference in 15km-TT performance after CON or BFR. In Study 2, using a repeated measures design, participants performed an acute bout of either BFR or CON. Muscle biopsies were taken before and after exercise to examine the activation of signalling pathways regulating angiogenesis and mitochondrial biogenesis. Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ increased by a similar extent after CON and BFR. There was no difference in the magnitude of increase in PGC-1α, VEGF and VEGFR-2 mRNA expression between protocols, however HIF-1α mRNA expression increased (P = 0.04) at 3 h only after BFR. We have demonstrated the potency of combining BFR with SIT in increasing $\dot{V}O_{2max}$ in trained individuals, however this did not translate to an enhanced exercise performance. SIT alone did not induce any observable adaptation. Although the mechanisms are not fully understood, we present preliminary evidence that BFR led to enhanced HIF-1α mediated cell signalling.

Introduction

Increasing the volume of submaximal endurance training does not appear to promote improvements in exercise capacity or maximal oxygen consumption ($\dot{V}O_{2max}$) in well-trained endurance athletes (Hickson *et al.*, 1981; Denis *et al.*, 1982; Costill *et al.*, 1988). In contrast, trained cyclists who supplement their aerobic base training with high-intensity interval training (HIT) demonstrate improvements in endurance capabilities (Lindsay *et al.*, 1996; Westgarth-Taylor *et al.*, 1997; Weston *et al.*, 1997) and as such HIT (e.g. involving longer duration intervals of approximately 1 - 8 min at sub-maximal exercise intensities) regularly features in many athletes' structured training programmes. Another example of HIT (sometimes called sprint interval training; SIT) comprises of 4-6 × 30 s 'all-out' maximal efforts interspersed by ~4 min passive recovery. Recent evidence demonstrates this type of training is an effective strategy to induce rapid increases in oxidative capacity and associated endurance capacity in healthy active and sedentary individuals (Burgomaster *et al.*, 2005, 2006, 2008; Gibala *et al.*, 2006) however the efficacy of this type of low volume sprint interval training model has yet to be established in trained individuals.

Despite the observed benefits of different types of HIT in promoting improvements in exercise capacity and $\dot{V}O_{2max}$ in trained individuals, there is increasing scope to identify and adopt novel, low volume training methods that result in greater adaptive responses. One potential strategy to enhance endurance adaptation would be to combine exercise with blood flow restriction (BFR). For example, one-legged endurance exercise (45 min at approximately 50% one-legged $\dot{V}O_{2peak}$ for four weeks, four times per week) performed with moderate blood flow restriction (~ 20 %) has been demonstrated to increase exercise capacity and $\dot{V}O_{2max}$ above that performed with a normal blood supply (Sundberg *et al.*, 1993). More recently, BFR using blood pressure cuffs combined with low load resistance training has

become a popular approach for enhancing adaptations in both skeletal muscle (Burgomaster *et al.*, 2003; Fujita *et al.*, 2007) and the peripheral vasculature, in particular within the conduit arteries and capillary bed (Hunt *et al.*, 2013)

Both HIT and SIT has been shown to enhance skeletal muscle capillary growth (Jensen *et al.*, 2004; Cocks *et al.*, 2013, respectively), a critical adaptation to enhance oxygen and substrate delivery. Exercise-induced capillary growth (angiogenesis) is a highly complex process, mediated by signal transduction pathways involving several transcription and angiogenic growth factors including hypoxic inducible factor 1 α (HIF-1α), PGC-1α and the primary capillary growth factor vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Chinsomboon *et al.*, 2009; Geng *et al.*, 2010). These molecular pathways are activated in response to different physiological signals (e.g. hypoxia, shear stress, mechanical and metabolic stress) that are specific to the nature of the exercise stimulus (Egginton, 2009). The addition of BFR, for example, is likely to increase tissue hypoxia, thereby activating HIF-1α mediated signalling to induce transcription of target genes involved in angiogenesis and energy metabolism (Taylor, 2008).

Therefore, there were two aims to the present investigation. Firstly, we assessed the potency of combining SIT (repeated 30 s maximal sprint cycling) with BFR in enhancing maximal aerobic physiology and performance in trained participants who already possess enhanced physiological performance capabilities. We hypothesised that BFR combined with SIT would result in a greater increase in VO_{2max} and 15km TT performance than SIT alone. Secondly, we explored the potential mechanisms of adaptation by measuring the expression of key signalling proteins and genes involved in skeletal muscle remodelling that contribute to enhanced aerobic physiological capabilities (i.e. angiogenesis and mitochondrial biogenesis).

Methods

Ethical approval

All experimental procedures were approved by the Loughborough University Ethics Advisory Committee and conformed in all respects with the Declaration of Helsinki. Participants were fully informed of the risks and discomforts associated with all experimental trials before providing written, informed consent.

Participants

A total of 28 healthy trained males who were cycling 120 ± 66 km per week volunteered to participate in the two studies (two of these participants took part in both studies). All completed a medical questionnaire and biopsy screening document prior to participation to mitigate for maximal exercise and biopsy contraindications. Participants did not have a history of neuromuscular, haematological or musculoskeletal abnormalities and were not using pharmacological treatments during the study period.

Experimental design

Study 1 – Training study. Using a between groups design, participants performed four weeks of sprint interval training either combined with post-exercise blood flow restriction (BFR) or sprint interval training alone (CON). Twenty participants took part in this study. There was no significant difference ($P > 0.05$) in pre-training age (27 ± 7 vs. 26 ± 5 yrs), height (181 ± 9 vs. 178 ± 8 cm), body mass (75.5 ± 8.4 vs. 74.1 ± 7.4 kg) and all other baseline outcome measures (Table 2) between the two intervention groups, respectively (CON, n = 10 vs. BFR, n = 10). Physiological ($\dot{V}O_{2max}$) and performance (15-km cycling time trial) assessments were made before and after training.

Study 2 – Acute study. Using a repeated measures design, participants performed an acute bout of sprint interval training either combined with post-exercise blood flow restriction (BFR) or sprint interval training alone (CON). Eight participants (age, 32 ± 7 yrs; height, 180 ± 10 cm; body mass, 75.3 ± 9.1 kg; $\dot{V}O_{2max}$, 4.3 ± 0.4 L.min^{-1}) took part in this study. Muscle biopsies were obtained before, immediately post- exercise and 3 h post-exercise.

Experimental protocols

Study 1 – Training study

Participants were initially familiarized to the testing and training procedures during preliminary visits, before being randomly assigned to either the control (CON) or blood flow restricted (BFR) training intervention. Pre-training outcome measures were assessed on two occasions separated by at least 2 days. Post-training outcome measures were assessed in the same order, each separated by 2 days within 1 week. Laboratory conditions during pre- and post-training measurements remained constant (19-21°C, 40-50% humidity) and all measurements were conducted at the same time of day for each participant. Ergometer saddle and handle bar dimensions recorded for each participant during preliminary testing were standardised for all post-training measures. Participants recorded dietary intake and physical activity performed during the 24 h prior to each of their pre-training tests and replicated these dietary and activity patterns for the 24h prior to all post-training tests. Participants were instructed to refrain from strenuous exercise 24h prior to all testing visits and from ingesting alcohol and caffeine during the 48 h preceding testing.

Pre and post training outcome measures

All pre and post training measures were conducted on an SRM cycle ergometer, calibrated according to the manufacturer guidelines (Schroberer Rad McBtechink, Weldorf, Germany).

Participants performed an incremental test to exhaustion to establish maximal oxygen uptake ($\dot{V}O_{2max}$) and maximal aerobic power (MAP). Participants began cycling, at a freely chosen, constant pedal cadence for 5 min at 120 W, after which power increased 20 W every 60 s. Pulmonary gas exchange was measured breath by breath throughout exercise (Oxycon Pro, Carefusion, UK). $\dot{V}O_{2max}$ and MAP were defined as the highest $\dot{V}O_2$ and power output achieved for a 30 and 60s period during the test, respectively.

The cycling time trial (15km-TT) involved completing 15km as quickly as possible. Except for total distance covered, no visual or verbal technical or motivational feedback was given. To better resemble a time trial on the road, the ergometer was placed into 'hyperbolic mode' enabling the participants to manually select their own gear, pedal cadence and thus power output throughout. Power output was recorded (2 Hz sample rate) and averaged throughout the entire duration of the 15km-TT performance trials. Participants consumed water *ad libitum* during their familiarisation trial with the volume and timings of consumption recorded and replicated during subsequent trials. Prior to commencing training, participants completed a total of three 15km-TT efforts each separated by a week, to establish familiarity. Reproducibility for the 15km-TT (i.e. the coefficient of variance which expresses the typical error as a percentage (Hopkins, 2000)) when performed at the same time of day and having consumed an identical diet for the 24 h preceding each trial was determined between the 2nd and 3rd TT efforts and was 4.9 %. The 3rd TT effort was reported as the pre-training outcome measure.

Exercise training

Participants completed a four-week supervised sprint interval training programme (2 sessions per week). Each training session consisted of repeated 30 s maximal sprint cycling bouts performed on a mechanically braked cycle ergometer (SE-780 50, Monark, Stockholm,

Sweden) against a manually applied resistance equivalent to 0.075 kg/kg body mass. The training was progressive whereby all participants performed a total of 4, 5, 6 and 7 maximal 30 s cycling bouts in weeks 1, 2, 3 and 4, respectively, with each bout separated by a 4.5 min recovery period. In CON participants remained seated and stationary on the ergometer and refrained from pedalling during recovery between each sprint. At times participants would get out of the saddle and stretch their legs and pedal backwards but this was minimised. In the BFR condition participants immediately dismounted the ergometer after each sprint and lay supine on a couch, upon which they were subjected to lower limb blood flow restriction (within 15s of each sprint). This was achieved by rapidly applying a pneumatic pressure cuff (Hokanson SC12L) as high up as possible on the proximal portion of each thigh, inflated (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA) to a pressure of ~ 130 mmHg for 2 min. After 2 min the cuff was rapidly deflated and the participants remained in a supine position for a further 2 min. The participants subsequently re-mounted the ergometer in time for the subsequent sprint which began precisely 4.5 min after the previous sprint ended. The cuff pressure of 130 mmHg was used following preliminary work that demonstrated this was the highest cuff pressure that could be tolerated in combination with the current sprint training protocol. This pressure was kept constant through the four-week training period. Participants were instructed to continue normal dietary practices throughout the training period and were instructed to complete daily training diaries to ensure that non-prescribed training remained constant throughout the entire study. Participants performed no other interval training during the study period and were instructed to replicate all other non-prescribed weekly training to ensure the SIT was the only training change experienced. Pre training measurements of peak power output (PPO) and mean power output (MPO) were obtained from the first training session after a standardised warm-up. Post-

training PPO and MPO were taken from the best of two maximal sprints performed separately during the week of post training outcome measurements.

Study 2 – Acute study

Preliminary testing

Participants reported to the laboratory on three separate occasions prior to their first experimental trial. On their first visit they performed an incremental test to exhaustion as outlined in Study 1 to establish their $\dot{V}O_{2max}$. They also performed a series of randomised maximal sprints (<12 s) on a SRM cycle ergometer at multiple fixed cadences (90, 100, 110, 120, 130 rpm) which was achieved by placing the ergometer into 'isokinetic mode'. These were performed to determine each participant's optimal cadence for peak power output (PPO) which was subsequently used as the fixed cadence at which each participant performed their experimental trials. On two subsequent visits, participants were familiarised with the experimental protocols.

Experimental protocol

Participants attended the laboratory in the morning (~ 0800 h) of each experimental trial following an overnight fast. On arrival, participants rested supine for 20 min whilst muscle biopsy sites were prepared, before a resting biopsy sample was obtained. Participants then performed a standardised warm-up, consisting of cycling at 120 W for 5 minutes. Immediately after the warm-up, they performed 4 × 30 s 'all out' sprints at the previously determined fixed pedal cadence, each sprint separated by 4.5 minutes of recovery. In CON participants remained seated and stationary on the ergometer between each sprint. In BFR participants immediately dismounted the ergometer after each sprint and lay supine on an adjacent couch and subjected to the BFR procedure as described in Study 1. At the cessation

of each protocol, participants dismounted the ergometer, and were helped onto an adjacent couch where a muscle biopsy was taken within 1 min. Participants then rested passively before having a final muscle biopsy 3 h post-exercise. Muscle biopsies were obtained at these time points as mRNA expression for the genes of interest measured have previously been shown to peak 1-6 h post intense exercise (Hoier *et al.*, 2012, 2013*a*, 2013*b*; Bartlett *et al.*, 2012) and post-exercise phosphorylation of p38MAPK$^{Thr180/Tyr182}$ has previously been demonstrated to peak and return to baseline within this time-course (Gibala *et al.*, 2009; Bartlett *et al.*, 2012).

Participants recorded all food consumed and physical activity during the 24 h prior to their first experimental trial and were instructed to replicate these dietary and activity patterns prior to their second experimental trial, which were separated by 14-21 days. They were also instructed to refrain from ingesting alcohol and caffeine during the 48 h preceding each trial. Whilst the consumption of food was prohibited at all times during each experimental trial, water was consumed *ad libitum*. Laboratory conditions remained constant (19-21°C, 40-50% humidity) for both experimental trials.

Muscle sampling and analysis

Muscle biopsies were obtained from the medial portion of the vastus lateralis muscle under local anaesthesia (1% lidocaine) using the micro-biopsy technique (Acecut 11G Biopsy Needle, TSK, Japan). All muscle samples were obtained through separate incisions >2 cm apart with two samples (~ 30 mg each) taken from each incision at each time point. Muscle samples were immediately frozen and stored at -80 °C until further analyses.

Western Blotting. Approximately 20 mg of frozen muscle was ground to powder under liquid nitrogen using a laboratory grade pestle and mortar before being homogenised in 120 µl of ice cold lysis buffer (25 mM Tris/HCl [pH 7.4], 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1

mM EDTA, 10 mM Na-Pyrophosphatase, 1 mM Na_3VO_4, 0.27 M sucrose, 1% Triton X-100, 0.1% 2-mercaptoethanol) supplemented with a Pierce™ Protease Inhibitor Tablet (Thermo Scientific, UK). Homogenates were centrifuged at 13,500*g* for 10 min at 4˚C and the supernatant collected. Protein content of the supernatant was determined using a Pierce™ 660 Protein Assay (Thermo Scientific, UK). Each sample was solubilized for 5 min at 100˚C with an equal volume of sample buffer containing 1 M Tris-HCl (pH 6.8), 8% glycerol, 10% sodium dodecyl sulphate, 0.4% 2-β-mercaptoethanol and 0.05% bromophenol blue. For each blot a negative control was loaded along with 10 µg of each sample and then separated (~2 h at ~100 V) in Tris-glycine running buffer using self-cast 4% stacking and 10% separating polyacrylamide gels. Gels were transferred wet onto nitrocellulose membranes for 2 h at 35 mA in a 1 × transfer buffer (0.3% Tris base, 1.4% glycine, 20% methanol). Membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBST: 0.19 M Tris [pH 7.6], 1.3 M NaCl, 0.1% Tween-20) with 5% non-fat blocking grade milk. Membranes were washed for 3 x 5 min in TBST before being incubated overnight at 4˚C with anti-phospho$^{Thr180/Tyr182}$ and anti-total p38MAPK antibody (Cell Signalling, UK), at a concentration of 1:1000 in 1 X TBST. The following morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with anti-species horseradish peroxidise-conjugated secondary antibody (Bio-Rad, UK) for 1 h at room temperature. After a further 3 x 5 min washes in TBST, membranes were saturated in chemiluminescence (SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA) for 5 min prior to exposure. Membranes were visualised using image analysis (ChemiDoc™ XRS+, Bio-Rad, Herts, UK), and band densities determined (Quality One 1-D analysis software v 4.6.8, Bio-Rad, Herts, UK). Samples from each participant for both exercise protocols were run on the same gel and all gels were run in duplicate to verify responses. Pre-exercise values of phosphorylation

relative to total for each participant were normalised to 1 with post-exercise and 3 h post-exercise values subsequently expressed as fold-change relative to pre-exercise values.

Real-time RT-PCR. One-step quantitative RT-PCR was used to determine skeletal muscle mRNA levels of genes of interest. Primer sequences (Table 1) were designed by Sigma-Aldrich (Sigma-Aldrich Co. Ltd., Haverhill, UK) ideally with 40-60% GC content and spanning exon-exon boundaries. Primer specificity was determined by performing BLAST and melt curve analysis at the end of each PCR run. Total RNA was isolated from muscle biopsies (~ 30 mg) using a pestle and mortar and TRIzol® reagent (Life Technologies/Invitrogen, USA), according to the manufacturer's protocol. Sample RNA concentration (232 ± 73 ng/µl) and purity (260/280: 1.9 ± 0.1) was confirmed using spectrophotometry (Nanodrop) before being stored at -80 °C for future use. 20 µl PCR reactions were made up as follows in a 96 well plate; 70 ng of RNA in 9.5 µl of nuclease free water, 0.2 µl of Quantifast Reverse Transcriptase mix (Qiagen, Crawley, UK), 0.15 µl of both forward and reverse primers at 100 µM concentrations, and 10 µl of SYBR green mix (Qiagen). All reactions were performed in triplicate. Once PCR plates were prepared, they were transferred to the mx3005p qPCR cycler (Stratagene MX3005P, Agilent Technologies, Berkshire, UK), which was programmed to perform the following steps; 50 °C for 10 minutes (reverse transcription), followed by a 5 minute hold at 95°C, and then 40 cycles at 95°C for 10 seconds and 60°C for 30 s. Fluorescence was detected at the end of each cycle, and expression levels were determined using the $2^{-\Delta\Delta Ct}$ method using RNA polymerase II (RPII) as the reference gene. Post-exercise values are reported as a fold-change relative to pre-exercise values.

Statistics

In Study 1 baseline outcome measures and training data were analysed using independent t-tests. Subsequent analysis was performed with two-factor repeated measures analysis of variance (ANOVA) with one within factor (time: pre versus post) and one between factor (group: CON versus BFR). In Study 2 protein phosphorylation and mRNA data were analysed using a two-way repeated measures ANOVA. Where significant main effects were observed, Bonferroni corrected post-hoc t-tests were used to locate differences. Data are presented as mean ± SD unless stated otherwise. Significance was accepted at $P < 0.05$.

Results

Study 1 – Training study

Participants completed 100% of the assigned training sessions without any complications. Prior to training, there was no difference in any variable between the two training groups (Table 2). The average power output developed over the 8 training sessions was greater (t-test $P < 0.01$) in CON (642 ± 10W) compared to BFR (618 ± 11W). Consequently, the total work done throughout the training was greater (t-test $P < 0.01$) in CON (106 ± 22 kJ) compared to BFR (102 ± 21 kJ).

Physiological and performance variables measured before and after CON and BFR are presented in Table 2. Absolute and relative $\dot{V}O_{2max}$ (Figure 1) increased after BFR but were unchanged after CON (interaction; both $P = 0.01$). There was a trend for absolute and relative MAP (Figure 1) to increase after BFR, with no changes after CON, however these did not reach statistical significance (interaction; $P = 0.11$ and $P = 0.09$, respectively). Absolute and relative sprint PPO increased (main effect for time; both $P = 0.02$) but there were no difference between training groups. There were no changes in absolute or relative sprint MPO after CON and BFR. 15km-TT performance time (Figure 1) and average power output during the TT were unchanged after CON and BFR.

Study 2 – Acute study

The optimal cadence for PPO was 122 ± 13 revs.min^{-1}. There was no difference (t-test $P = 0.91$) in PPO achieved during BFR and CON (1147 ± 171 W vs 1149 ± 179 W, respectively). Total work done was similar (t-test $P = 0.11$) between protocols (BFR; 67.1 ± 9.8 vs. CON; 68.3 ± 10.4 kJ).

p38MAPK activation. Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ increased (main effect for time; $P = 0.02$) 3.2- and 4.1-fold immediately following CON and BFR, respectively, before returning to baseline at 3 h post-exercise (Figure 2). The magnitude of phosphorylation immediately post-exercise was not different between protocols (interaction; $P = 0.52$).

Angiogenic mRNA expression. mRNA expression data is presented in Figure 3. PGC-1α, VEGF, and VEGFR-2 increased (main effects for time; all $P = 0.01$) at 3 h in both CON and BFR. There was, however, no difference in the magnitude of fold-change for either of these genes between protocols. HIF-1α mRNA expression increased (interaction; $P = 0.04$) at 3 h only after BFR. There was no change in eNOS (interaction; $P = 0.25$), MMP-9 (interaction; $P = 0.71$) or Ang2 (interaction; $P = 0.52$) mRNA expression in response to either protocol.

Discussion

This study has demonstrated the potency of combining blood flow restriction with sprint interval training (SIT) in increasing $\dot{V}O_{2max}$ in trained individuals. The mechanisms responsible for this adaptive response are unclear however the current study presents preliminary evidence to suggest enhanced skeletal muscle remodelling, in particular capillary density, could occur with BFR compared to SIT alone through enhanced hypoxia induced cell-signalling, given the greater expression of the angiogenic growth factor HIF-1α.

In the present study, $\dot{V}O_{2max}$ increased by ~4.5% in response to SIT with BFR, compared with 0.7% in CON. The potency of combining BFR in enhancing oxygen uptake and the adaptive response to other forms of exercise has previously been demonstrated. For example, one-legged cycle training whereby blood flow was restricted during exercise by approximately 20% through the use of a pressure chamber (Sundberg & Kaijser, 1992) has previously been demonstrated to promote a greater increase in one-legged $\dot{V}O_{2peak}$ over that performed with normal blood supply (Sundberg *et al.*, 1993). Of course, the use of such pressure chambers is limited by accessibility and ease of use, whereas the use of blood pressure cuffs placed on the proximal portion of the legs and a rapid inflation system seems effective in providing a fixed pressure for a set duration. The feasibility of combining BFR with whole body exercise, specifically SIT has also been demonstrated. This is of particular relevance to well-trained cyclists where there is a requirement to maintain an appropriate load or training intensity as part of a structured training programme. In pilot work, combining SIT and BFR concurrently proved impractical and even at moderate cuff pressures (100 mmHg) this combination was not tolerable. Either way, this type of training represents a novel way to enhance the adaptive responses in well trained individuals.

There was no improvement in $\dot{V}O_{2max}$ with SIT alone in our group of trained individuals (CON). The effects of SIT on $\dot{V}O_{2max}$ in less-trained individuals is inconsistent, with some studies demonstrating increases in response to training (Burgomaster *et al.*, 2006, 2008; Cocks *et al.*, 2013) and others not (Burgomaster *et al.*, 2005). Indeed, previous investigations into the effects of HIT (that involve longer duration intervals at sub-maximal intensities rather than 'all out' maximal sprints) have demonstrated positive adaptive responses in indices of aerobic performance (e.g. $\dot{V}O_{2max}$, lactate threshold) in well-trained cyclists with pre-training $\dot{V}O_{2max}$ values of ~ 65 $mL.min^{-1}.kg^{-1}$ (Laursen & Jenkins, 2002; Laursen *et al.*, 2005). Differences in the $\dot{V}O_{2max}$ response to training between the aforementioned studies could be attributable to a number of factors such as differences in the duration, intensity and frequency of the interval training stimulus, as well as the total training volume prescribed.

The main determinants of $\dot{V}O_{2max}$ are complex. Although it is generally accepted that the vascular bed is not a limiting factor for oxygen transport during one legged knee-extensor exercise (Andersen & Saltin, 1985), performing cycling exercise in which a larger muscle mass is recruited stresses both central and peripheral limitations in oxygen delivery and uptake (Poole & Richardson, 1997). As well as the important role the capillary network has on increasing oxygen delivery and reducing diffusion gradients, it also facilitates greater removal of metabolic end products which sustains exercise tolerance (Joyner & Coyle, 2008). Moreover, an increased capillary density specific to type II muscle fibres will improve performance and recovery from maximal and 'all out' exercise (Tesch & Wright, 1983) during which these fibres are preferentially recruited (Greenhaff *et al.*, 1994). Since previous work involving BFR has demonstrated this model as an effective stimulus for enhancing capillary growth (Esbjörnsson *et al.*, 1993) we sought to examine the potency of this type of exercise for enhancing capillary growth by measuring the acute activation and transcription

of signalling proteins and genes involved in mediating exercise-induced angiogenesis. The expression of PGC-1α, VEGF and VEGFR-2 mRNA increased to a similar extent in both exercise conditions suggesting that SIT has the potential in its own right for inducing capillary growth, as has been demonstrated previously in sedentary individuals (Cocks *et al.*, 2013). Of note, there was a greater HIF-1α mRNA expression in the early recovery after BFR, but not CON. HIF-1α is seen as a key regulator of the tissue to hypoxia and thus metabolic stress (Semenza *et al.*, 2006; Semenza, 2006), during which it is stabilized and functions in regulating angiogenesis (Lee *et al.*, 2004). Indeed, a reduction in oxygen tension results in an accumulation of HIF-1α (the oxygen sensing subunit) protein and translocation into the nucleus for targeted activation of VEGF in human skeletal muscle (Ameln *et al.*, 2005). Acute exercise results in an increase in skeletal muscle HIF-1α mRNA expression provided that the exercise intensity is sufficient and/or the tissue is exposed to an hypoxic stimulus (Vogt *et al.*, 2001; Zoll *et al.*, 2006). Thus, the greater HIF-1α mRNA expression after BFR likely reflects a greater reduction in the intracellular partial pressure of oxygen in the muscle during the restriction periods and an increased potential for activation of downstream HIF-1α dependent pathways. Whilst this tentatively suggests BFR provides an intensified stimulus for hypoxia-mediated angiogenesis further work is clearly required to support this hypothesis. In particular, the inclusion of muscle protein analysis and immuno-histochemical techniques to evaluate direct changes in capillary density is necessary.

$\dot{V}O_{2max}$ has a clear importance for exercise capacity, as does the performance $\dot{V}O_2$ and power/velocity at lactate threshold which is also directly influenced by muscle capillary density (Joyner & Coyle, 2008). These measurements, however, are not necessarily direct assessments of performance *per se*. We chose to assess exercise performance with a 15km self-paced time trial (TT). Despite the improvements in $\dot{V}O_{2max}$ with BFR, this did not translate to an improved TT performance, which did not improve with SIT alone either.

Whilst this is perhaps surprising given HIT has previously been demonstrated to improve TT performance in well trained athletes (Laursen *et al.*, 2005), the TT distance needs to be considered when considering performance improvements. For example, performance during self-paced exercise is predominantly limited by central or peripheral factors with a greater degree of peripheral fatigue evident after shorter high-intensity (approximately 6 min) TTs and increased contribution of central fatigue after longer lower-intensity TTs (>30 min) (Thomas *et al.*, 2015). It is also plausible that our 15km TT may have lacked sensitivity to at least partly reflect an increased $\dot{V}O_{2max.}$ Nevertheless, we feel it is important that future studies continue to include TT tests of performance to better inform the application of training interventions to athletic populations. There were also slight trends towards significance in some of the performance outcome measures (MAP and MPO; Table 1) although the observed statistical power for each outcome measure were rather moderate (MAP, ~0.4; MPO, ~0.3). It is feasible that with greater participant numbers these measurements would reach statistical significance. Nevertheless, gains in the region of 2.9% to 4.4% in these outcome measures in the BFR group compared to more marginal changes of 0.2% to 0.3% in the CON group, respectively, are more meaningful for athletic performance coaches and scientists rather than achieving a traditional statistical significance.

The present study is not without limitations. The lack of muscle biopsies before and after training for the assessment of capillarisation, limits the development of firmer conclusions that such a training stimulus manifests itself in such skeletal muscle remodelling. Whilst there are limitations in predicting chronic adaptations from acute gene expression data, previous studies have demonstrated that the acute angiogenic response can help to inform the magnitude of capillary growth with training (Hellsten *et al.*, 2008; Høier *et al.*, 2010; Hoier *et al.*, 2012). Temporal factors relating to the timing and number of biopsies need to be considered when making inferences based on transient molecular responses to acute exercise

interventions. Although the half-lives of some of the genes of interest are very short e.g. HIF-1α is approximately 5 min (Berra *et al*., 2001) and VEGF is less than 1 hour, albeit more prolonged in hypoxia (Shima *et al*., 2995), the fact that it has been previously shown that mRNA expression for these genes have been shown to peak between 1-6 h post intense exercise (Hoier *et al.*, 2012, 2013*a*, 2013*b*; Bartlett *et al.*, 2012) suggests that the timing of our post exercise biopsies were appropriate. Finally, the current study did not involve the quantification of actual percentage of blood flow restriction induced by the cuff pressure of 130 mmHg. This chosen occlusion pressure, resulting from preliminary work in our laboratory, was the highest cuff pressure that could be tolerated in combination with SIT. Previous work in our laboratory has demonstrated this cuff pressure around the mid-thigh restricts resting blood flow to the popliteal artery by ~76% (Hunt et al. unpublished data).

In conclusion, we have demonstrated the potency of combining BFR with SIT in increasing $\dot{V}O_{2max}$ in trained athletes, although this did not translate to an enhanced exercise performance. SIT alone did not induce any observable adaptation. Although the potential mechanisms are not fully understood, we present evidence of an enhanced HIF-1α mediated cell signalling compared to SIT alone.

Additional information

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

All authors contributed to the conception and design of the experiment, interpretation of data and writing of the manuscript. Conor Taylor and Richard Ferguson contributed to the collection and analysis of data. All authors read and approved the final manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This project was partially supported by a grant from the English Institute of Sport.

Acknowledgements

The authors would like to thank all the participants for their commitment and effort. We also thank Dr Neil Martin for help with muscle tissue analysis as well as Rachel Malcolm, Xin Hui Aw Yong, Joseph Mitchell and George Antoniades for their technical assistance.

References

Ameln H, Gustafsson T, Sundberg CJ, Okamoto K, Jansson E, Poellinger L & Makino Y (2005). Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* **19,** 1009–1011.

Andersen P & Saltin B (1985). Maximal perfusion of skeletal muscle in man. *J Physiol* **366,** 233–249.

Bartlett JD, Hwa Joo C, Jeong T-S, Louhelainen J, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B & Morton JP (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *J Appl Physiol* **112,** 1135–1143.

Berra E, Richard DE, Gothie E, Pouyssegur J (2001). HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1a degradation. *FEBS Lett* **491**, 85-90.

Burgomaster K a, Heigenhauser GJF & Gibala MJ (2006). Effect of short-term sprint interval training on human skeletal muscle carbohydrate metabolism during exercise and time-trial performance. *J Appl Physiol* **100,** 2041–2047.

Burgomaster K a, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL & Gibala MJ (2008). Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* **586,** 151–160.

Burgomaster K a, Hughes SC, Heigenhauser GJF, Bradwell SN & Gibala MJ (2005). Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol* **98,** 1985–1990.

Burgomaster K a, Moore DR, Schofield LM, Phillips SM, Sale DG & Gibala MJ (2003). Resistance training with vascular occlusion: metabolic adaptations in human muscle. *Med Sci Sports Exerc* **35,** 1203–1208.

Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S & Arany Z (2009). The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle. *Proc Natl Acad Sci U S A* **106,** 21401–21406.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker T a, Tipton KD & Wagenmakers AJM (2013). Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *J Physiol* **591,** 641–656.

Costill DL, Flynn MG, Kirwan JP, Houmard JA, Mitchell JB, Thomas R & Park SH (1988). Effects of repeated days of intensified training on muscle glycogen and swimming performance. *Med Sci Sports Exerc* **20,** 249–254.

Denis C, Fouquet R, Poty P, Geyssant a & Lacour JR (1982). Effect of 40 weeks of endurance training on the anaerobic threshold. *Int J Sports Med* **3,** 208–214.

Egginton S (2009). Invited review: activity-induced angiogenesis. *Pflugers Arch* **457,** 963–977.

Esbjörnsson M, Jansson E, Sundberg CJ, Sylvén C, Eiken O, Nygren A & Kaijser L (1993). Muscle fibre types and enzyme activities after training with local leg ischaemia in man. *Acta Physiol Scand* **148,** 233–241.

Fujita S, Abe T, Drummond MJ, Cadenas JG, Dreyer HC, Sato Y, Volpi E & Rasmussen BB (2007). Blood flow restriction during low-intensity resistance exercise increases S6K1 phosphorylation and muscle protein synthesis. *J Appl Physiol* **103,** 903–910.

Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M & Yan Z (2010). PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *Am J Physiol Cell Physiol* **298,** C572–C579.

Gibala MJ, Little JP, van Essen M, Wilkin GP, Burgomaster K a, Safdar A, Raha S & Tarnopolsky M a (2006). Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *J Physiol* **575,** 901–911.

Gibala MJ, McGee SL, Garnham AP, Howlett KF, Snow RJ & Hargreaves M (2009). Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. *J Appl Physiol* **106,** 929–934.

Greenhaff PL, Nevill ME, Soderlund K, Bodin K, Boobis LH, Williams C & Hultman E (1994). The metabolic responses of human type I and II muscle fibres during maximal treadmill sprinting. *J Physiol* **478 (Pt 1,** 149–155.

Hellsten Y, Rufener N, Nielsen JJ, Høier B, Krustrup P & Bangsbo J (2008). Passive leg movement enhances interstitial VEGF protein, endothelial cell proliferation, and eNOS mRNA content in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **294,** R975–R982.

Hickson RC, Hagberg JM, Ehsani AA & Holloszy JO (1981). Time course of the adaptive responses of aerobic power and heart rate to training. *Med Sci Sports Exerc* **13,** 17–20.

Hoier 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. *J Physiol* **590,** 595–606.

Hoier B, Passos M, Bangsbo J & Hellsten Y (2013*a*). Intense intermittent exercise provides weak stimulus for vascular endothelial growth factor secretion and capillary growth in skeletal muscle. *Exp Physiol* **98,** 585–597.

Hoier B, Prats C, Qvortrup K, Pilegaard H, Bangsbo J & Hellsten Y (2013*b*). Subcellular localization and mechanism of secretion of vascular endothelial growth factor in human skeletal muscle. *FASEB J*; DOI: 10.1096/fj.12-224618.

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. *J Physiol* **588,** 3833–3845.

Hopkins WG (2000). Measures of reliability in sports medicine and science. *Sports Med* **30,** 1–15.

Hunt JEA, Galea D, Tufft G, Bunce D & Ferguson RA (2013). Time course of regional vascular adaptations to low load resistance training with blood flow restriction. *J Appl Physiol* **115,** 403–411.

Jensen L, Bangsbo J & Hellsten Y (2004). Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. **2,** 571–582.

Joyner MJ & Coyle EF (2008). Endurance exercise performance: the physiology of champions. *J Physiol* **586,** 35–44.

Laursen PB & Jenkins DG (2002). The scientific basis for high-intensity interval training: optimising training programmes and maximising performance in highly trained endurance athletes. *Sports Med* **32,** 53–73.

Laursen PB, Shing CM, Peake JM, Coombes JS & Jenkins DG (2005). Influence of high-intensity interval training on adaptations in well-trained cyclists. *J Strength Cond Res* **19,** 527–533.

Lee J-W, Bae S-H, Jeong J-W, Kim S-H & Kim K-W (2004). Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. *Exp Mol Med* **36,** 1–12.

Lindsay FH, Hawley JA, Myburgh KH, Schomer HH, Noakes TD & Dennis SC (1996). Improved athletic performance in highly trained cyclists after interval training. *Med Sci Sports Exerc* **28,** 1427–1434.

Poole DC & Richardson RS (1997). Determinants of oxygen uptake. Implications for exercise testing. *Sports Med* **24,** 308–320.

Semenza GL (2006). Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. *Exp Physiol* **91,** 803–806.

Semenza GL, Shimoda LA & Prabhakar NR (2006). Regulation of gene expression by HIF-1. *Novartis Found Symp* **272,** 2–8; discussion 8–14, 33–36.

Shima DT, Deutsch U, D'Amore PA (1995). Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. *FEBS Lett* **370**, 203-208.

Sundberg CJ, Eiken O, Nygren A & Kaijser L (1993). Effects of ischaemic training on local aerobic muscle performance in man. *Acta Physiol Scand* **148,** 13–19.

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

Taylor CT (2008). Mitochondria and cellular oxygen sensing in the HIF pathway. *Biochem J* **409,** 19–26.

Tesch PA & Wright JE (1983). Recovery from short term intense exercise: its relation to capillary supply and blood lactate concentration. *Eur J Appl Physiol Occup Physiol* **52,** 98–103.

Thomas K, Goodall S, Stone M, Howatson G, St Clair Gibson A & Ansley L (2015). Central and peripheral fatigue in male cyclists after 4-, 20-, and 40-km time trials. *Med Sci Sports Exerc* **47,** 537–546.

Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R & Hoppeler H (2001). Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol* **91,** 173–182.

Westgarth-Taylor C, Hawley JA, Rickard S, Myburgh KH, Noakes TD & Dennis SC (1997). Metabolic and performance adaptations to interval training in endurance-trained cyclists. *Eur J Appl Physiol Occup Physiol* **75,** 298–304.

Weston AR, Myburgh KH, Lindsay FH, Dennis SC, Noakes TD & Hawley JA (1997). Skeletal muscle buffering capacity and endurance performance after high-intensity interval training by well-trained cyclists. *Eur J Appl Physiol Occup Physiol* **75,** 7–13.

Zoll J, Ponsot E, Dufour S, Doutreleau S, Ventura-Clapier R, Vogt M, Hoppeler H, Richard R & Flück M (2006). Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts. *J Appl Physiol* **100,** 1258–1266.

Figure 1. Individual values of absolute VO_{2max} (A), relative VO_{2max} (B), MAP (C) and 15km-TT performance (D) for all participants before and after the 4-wk CON and BFR training interventions.

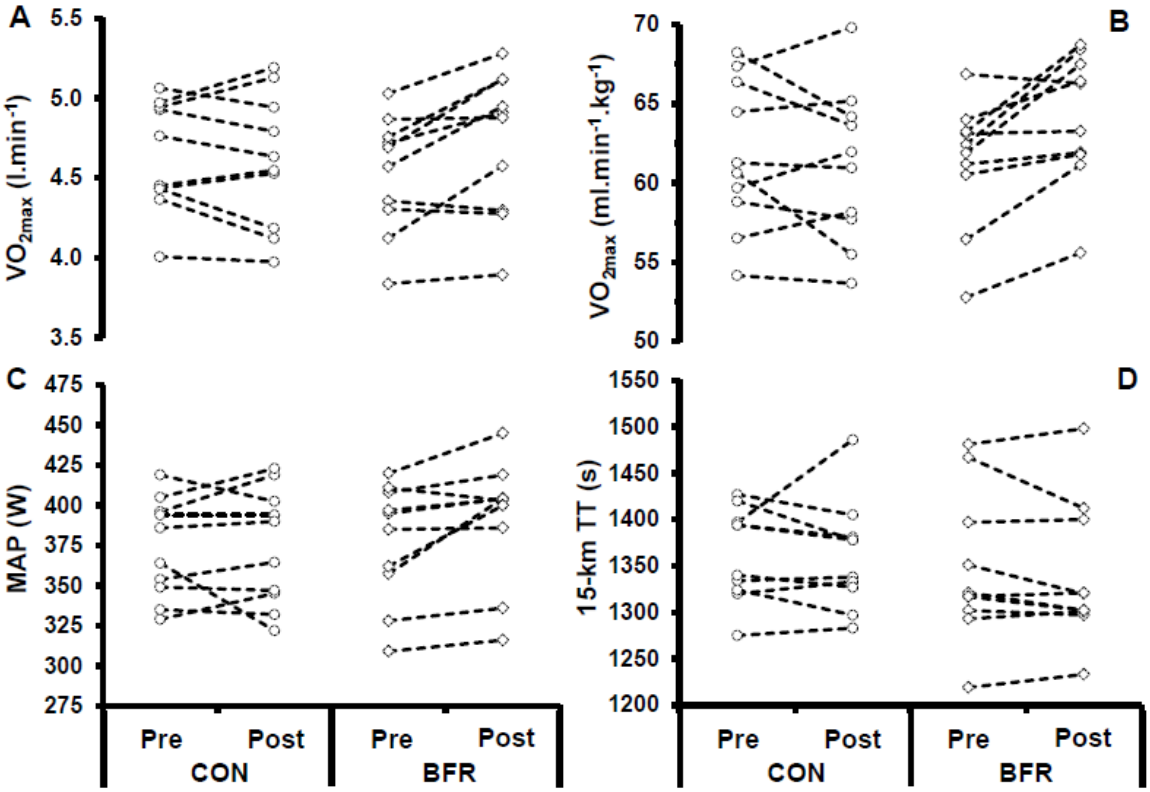

Figure 2. Phosphorylation of p38MAPK$^{Thr182/Tyr182}$ expressed relative to total p38MAPK immediately before (Pre), after (Post) and 3 h after (3h) the CON and BFR protocols. Each subject's Pre values have been normalised to 1 (hence no error bars are shown for this time-point) such that Post and 3 h values are subsequently expressed as fold-change relative to pre-values. Values are means ± SE (n=8). * denotes significant difference from Pre (P < 0.05).

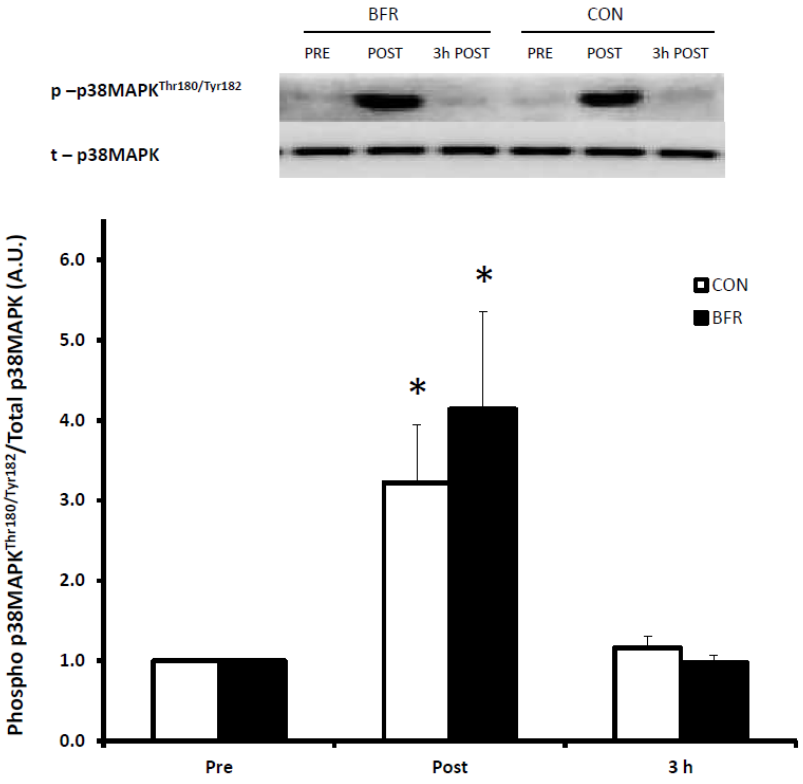

Figure 3. A) PGC-1α, B) VEGF, C) VEGFR-2, D) HIF-1α , E) MMP-9, F) eNOS, and G) Ang-2 mRNA expression immediately before (Pre), after (Post) and 3 h after (3h) the CON (open bars) and BFR (closed bars) protocols. Gene expression is normalised to RPII mRNA expressed relative to Pre. Values are means ± SE (n=8). * denotes significant difference from Pre ($P < 0.05$).

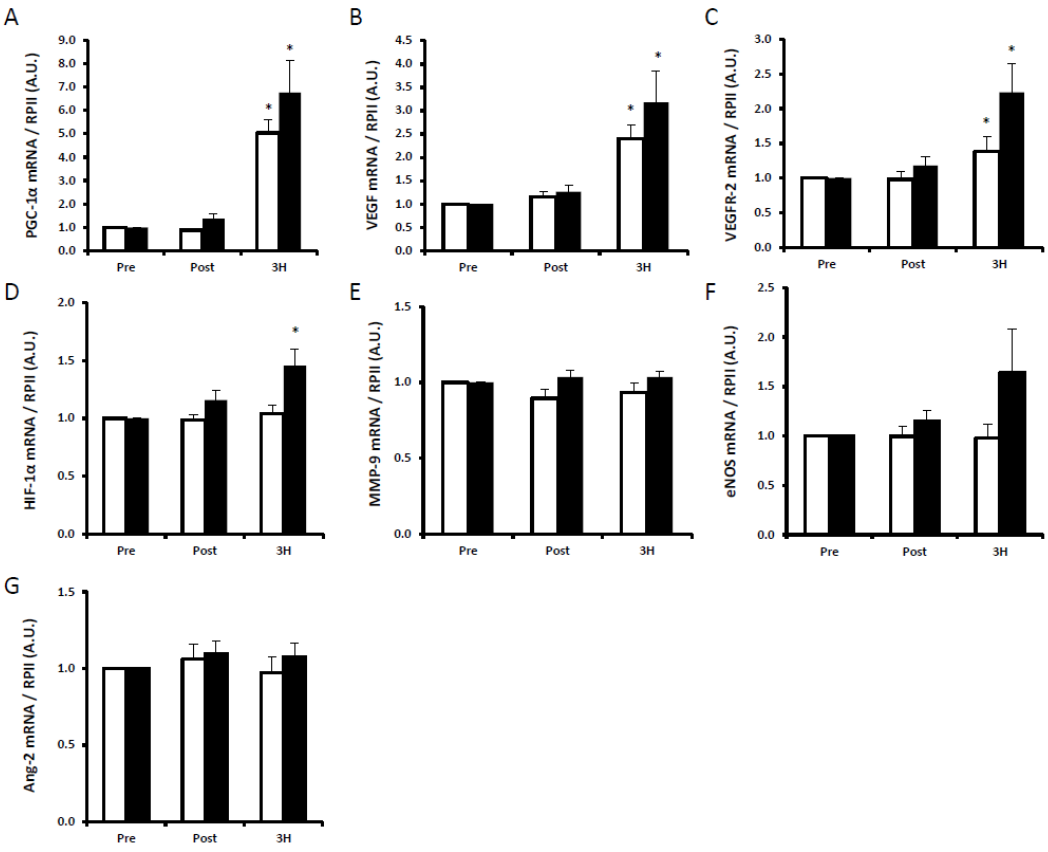

Table 1. Primers used in quantitative real-time PCR analysis

Target gene		Primer sequence	No. in Gene bank
VEGF	Forward	CTGCTCTACCTCCACCAT	NM_001171630
	Reverse	ATGAACTTCACCACTTCGT	
VEGFR-2	Forward	AGCAGGAATCAGTCAGTAT	NM_000459
	Reverse	TTACTTCTGGTTCTTCTAACG	
PGC-1α	Forward	CCTCTTCAAGATCCTGCTA	NM_013261
	Reverse	ACTCTCGCTTCTCATACTC	
eNOS	Forward	CAAGTTGGAATCTCGTGAA	NM_001160111
	Reverse	TGTGAAGGCTGTAGGTTAT	
MMP-9	Forward	GGCACCTCTATGGTCCTC	NM_004994
	Reverse	AGTAGTGGCCGTAGAAGG	
Ang2	Forward	GATTAGACAGAACACCTATGC	NM_001118887
	Reverse	CAGTGCTCAGAAGAATGC	
HIF-1α	Forward	TCACCTGAGCCTAATAGTC	NM_181054
	Reverse	AATCTGTGTCCTGAGTAGAA	
RPII	Forward	GAGTCAACGGATTTGGTC	NM_000938.1
	Reverse	GGTGGAATCATATTGGAACAT	

VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator- 1α; eNOS, endothelial nitric oxide synthase; MMP-9, membrane metalloproteinase-9; Ang2, angiopoietin-2; HIF-1α, hypoxic inducible factor-1α; RPII, RNA polymerase II

Table 2. Physiological and performance variables before and after CON and BFR training interventions (Study 1)

	CON			BFR			
	PRE	**POST**	**% change**	**PRE**	**POST**	**% change**	**ANOVA interaction P value**
Body mass (kg)	75.5 ± 8.4	75.7 ± 7.9	**0.3**	74.1 ± 7.4	74.0 ± 7.6	**0.3**	**0.55**
$\dot{V}O_{2max}$ **(l.min^{-1})**	4.6 ± 0.3	4.6 ± 0.4	**0.7**	4.5 ± 0.4	4.7 ± 0.4 *	**4.5**	**0.01**
$\dot{V}O_{2max}$ **(ml.min^{-1}.kg^{-1})**	61.8 ± 4.7	61.1 ± 4.9	**1.1**	61.2 ± 4.0	64.1 ± 4.1 *	**4.7**	**0.01**
MAP (W)	373 ± 31	374 ± 37	**0.2**	377 ± 37	392 ± 38	**3.8**	**0.11**
MAP (W.kg^{-1})	5.0 ± 0.4	5.0 ± 0.4	**0.2**	5.1 ± 0.3	5.3 ± 0.5	**4.4**	**0.09**
PPO (W)	1065 ± 214	1137 ± 259	**6.8**	938 ± 168	998 ± 203	**6.4**	**0.80**
PPO (W.kg^{-1})	14.0 ± 1.9	15.0 ± 2.9	**7.0**	12.6 ± 1.5	13.5 ± 2.3	**7.1**	**0.95**
MPO (W)	709 ± 99	711 ± 102	**0.2**	676 ± 97	696 ± 102	**2.9**	**0.19**
MPO (W.kg^{-1})	9.4 ± 0.8	9.4 ± 0.9	**0.3**	9.1 ± 0.7	9.4 ± 0.9	**3.3**	**0.14**
15 km-TT (s)	1363 ± 50	1361 ± 59	**0.1**	1347 ± 81	1339 ± 76	**0.6**	**0.66**
Ave TT power (W)	280 ± 26	288 ± 29	**2.8**	282 ± 39	285 ± 37	**1.1**	**0.53**

Values are mean ± SD. $\dot{V}O_{2max}$, maximal oxygen uptake; MAP, maximal aerobic power; PPO, peak power output during 30 s sprint; MPO, mean power output during 30 s sprint; TT, time trial. * $P < 0.05$, compared to PRE (Bonferroni corrected post-hoc t-test). All data are n = 10, except for Ave TT power which is n = 7 in each group.