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Blood flow-restricted training enhances thigh glucose uptake during exercise and muscle antioxidant function in humans

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ABSTRACT

This study examined the effects of blood-flow-restricted (BFR)-training on thigh glucose uptake at rest and during exercise in humans and the muscular mechanisms involved. Ten active men (~25 y; VO_{2max} ~50 mL/kg/min) completed six weeks of training, where one leg trained with BFR (cuff pressure: ~180 mmHg) and the other leg without BFR. Before and after training, thigh glucose uptake was determined at rest and during exercise at 25% and 90% of leg incremental peak power output by sampling of femoral arterial and venous blood and measurement of femoral arterial blood flow. Furthermore, resting muscle samples were collected. After training, thigh glucose uptake during exercise was higher than before training only in the BFR-trained leg ($p < 0.05$) due to increased glucose extraction ($p < 0.05$). Further, BFR-training substantially improved time to exhaustion during exhaustive exercise (11 ± 5% vs. CON-leg; $p = 0.001$). After but not before training, NAC infusion attenuated (~50–100%) leg net glucose uptake and extraction during exercise only in the BFR-trained leg, which coincided with an increased muscle abundance of Cu/Zn-SOD (39%), GPX-1 (29%), GLUT4 (28%), and nNOS (18%) ($p < 0.05$). Training did not affect Mn-SOD, catalase, and VEGF abundance in either leg ($p > 0.05$), although Mn-SOD was higher in BFR-leg vs. CON-leg after training ($p < 0.05$). The ratios of p-AMPK-Thr^{172}/AMPK and p-ACC-Ser^{79}/ACC, and p-ACC-Ser^{79}, remained unchanged in both legs ($p > 0.05$), despite a higher p-AMPK-Thr^{172} in BFR-leg after training (38%; $p < 0.05$). In conclusion, BFR-training enhances glucose uptake by exercising muscles in humans probably due to an increase in antioxidant function, GLUT4 abundance, and/or NO availability.

1. Introduction

Skeletal muscle is of significant importance for maintaining glucose homeostasis in man [1]. During exercise, muscle glucose uptake increases dramatically [2] due to an increase in glucose supply, transmembrane transport, and utilization within recruited muscle fibres [3]. In humans, exercise training raises both insulin- [4,5] and exercise-stimulated [6] muscle glucose uptake, which has been ascribed to a higher glucose extraction [6] and abundance of the glucose transporter isoform 4 (GLUT4) [5–9]. This may explain the remarkably high rate of glucose utilization and improved exercise performance after carbohydrate ingestion in well-trained men [10,11]. On the other hand, glucose metabolism is impaired in several disease states, including type-2 diabetes [12] and hypertension [13]. Several studies support the usefulness of high-intensity interval training for treating type-2 diabetes (e.g., lowering postprandial hyperglycaemia) [14–16]. However, there is a general concern about the safety of vigorous exercise for these patients due to an increased risk of cardiovascular events [17]. Thus, development of new strategies that can promote improvements in muscle glucose regulation in humans is of broad interest and significant relevance.

One possible strategy to facilitate improvements in muscle glucose regulation is to train with a reduced blood flow to exercising muscles, known as blood-flow-restricted (BFR)-training. BFR is typically induced by inflation of an occlusion cuff around the proximal portion of the exercising limbs. We have recently shown that a single interval exercise session with BFR elicits substantially greater increases in 5′AMP-activated protein kinase (AMPK) downstream signaling compared to a session without BFR in trained men [18]. In the same study, the session with BFR caused drastic fluctuations in muscle redox state (as assessed by near-infrared spectroscopy) and increased markers of oxidative damage [18], indicating high accumulation of reactive oxygen species (ROS) [19]. AMPK is a positive regulator of GLUT4 mRNA [20] and protein expression [21], whereas ROS activate AMPK in myocytes in vitro [22]. Thus, by modulating ROS levels and AMPK activity, BFR-training may be a potent stimulus to increase GLUT4 abundance, and thus glucose uptake, in human skeletal muscle. In another human study, BFR augmented exercise training-induced increases in nitric oxide (NO) bioavailability, as indicated by higher plasma nitrate concentration [23]. With the use of neuronal nitric oxide synthase (nNOS) inhibitors

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(e.g., L-NAME and L-NMMA), knock-out models, or NO precursors (e.g., L-arginine), studies in both mice [24–26] and humans [27–29] have demonstrated a role of NO in regulating contraction-stimulated muscle glucose uptake. Thus, an improved capacity for NO production could be another mechanism, by which BFR-training could accelerate glucose uptake by exercising skeletal muscle. In addition, the stimulating effect of BFR-exercise on AMPK signaling [18] and the demonstrated involvement of AMPK in insulin- and exercise-invoked glucose uptake [30,31], further support this possibility.

Several experiments in mice ex vivo showed an attenuating effect of the non-selective antioxidant, *N*-acetylcysteine (NAC), on muscle glucose uptake [24,32], indicating an increase in antioxidant function may depress muscle glucose uptake. As glucose is a key substrate for ATP resynthesis in exercising muscles [3], it may seem surprising that NAC postponed contraction-stimulated muscle fatigue in some human studies [33,34]. However, the effect of NAC on fatigue resistance in humans depends on contraction frequency [33] and thus the proportion of activated type-I and –II muscle fibres, which differ in their reliance on glucose uptake for ATP resynthesis [35]. Moreover, due to the short-term exhaustive nature [34] or low intensity (10 Hz) of contractions [33] in experiments where NAC improved fatigue resistance, factors other than restrictions in glucose uptake had likely more effect on fatigue development, such as disturbance of ion homeostasis, which under some experimental conditions is counteracted by NAC [36,37]. In contrast to the findings in mice, intravenous infusion of NAC was without effect on tracer (6,6-(2)H-glucose)-determined muscle glucose disposal during moderate-intensity exercise in recreationally-active men [38]. However, in the latter study, NAC failed to increase muscle antioxidant capacity, as indicated by unaltered reduced glutathione (GSH) concentration and GSH-to-oxidised GSH (GSSG)-ratio, which could explain the absence of an effect of NAC. Recently, we have shown that six weeks of BFR-training, performed as cycling intervals with BFR separated by periods with uninterrupted muscle blood flow, raised muscle GSH:GSSG ratio compared to training without BFR, and this was associated with a stimulating effect of NAC infusion on net potassium ion release from thigh muscles in humans [39]. Together with above indications in mouse skeletal muscle, these results indicate that interval training with BFR alters antioxidant function, which could modulate ROS and/or NO levels [40,41], and thus their impact on muscle glucose uptake.

Thus, the aim of the present study was to investigate the effects of BFR-training on thigh glucose uptake at rest and during exercise in humans and the muscular mechanisms involved. Specifically, the following hypotheses were tested: BFR-training 1) increases net thigh glucose uptake during exercise at both moderate and high intensity; 2) potentiates the attenuating effect of intravenous NAC infusion on thigh glucose uptake during exercise; and 3) elevates the abundance of endogenous antioxidant enzymes (Cu/Zn-SOD, Mn-SOD, GPX-1, and catalase), neuronal nitric oxide synthase (nNOS), and GLUT4 in skeletal muscle.

2. Methods

2.1. Ethical approval and participants

Thirteen men (age: 25 ± 4 y; height: 183 ± 6 cm; body mass: 83.6 ± 14 kg; VO_{2max}: 49.7 ± 5.3 $mL \cdot min^{-1} \cdot kg^{-1}$; mean ± SD) engaged in recreational activity two to three times per week were recruited for this study. The participants were non-smokers and free of medication and injury, and were instructed to keep their weekly training routine during the course of the study. Two subjects withdrew on the first experimental day, and another subject pulled out in the second week of the familiarisation period, because of the invasive procedures or lack of motivation, respectively. The weekly training routine of the participants who completed the study consisted of either two 1.5-h soccer training sessions (i.e., small-sided games, and/or high- and low-intensity drills with change of direction with and without the ball; n = 3), one 30-min session of continuous, intense running plus two swimming sessions (i.e., 20 min with various technical crawl drills, ending with 20 min of continuous crawl; n = 1), three sessions of combined body-loaded resistance training (i.e., 3 to 5 sets of 10–20 repetitions of squats, push-ups, pull-ups, and sit-ups) and aerobic training (i.e., 15 min of moderate-intensity cycling or running; n = 4), or two resistance-training sessions (i.e., 3 sets of 8–12 maximal repetitions of bench press, bent-over row, shoulder press, barbell-back squat, and hamstring curl; n = 2). All participants were informed about the benefits and possible risks associated with the study prior to providing oral and written informed consent. The Health Research Ethics Committee of the Capital Region of Denmark approved this study (approval no. H-16000377), which was performed in accordance with the 2013 Declaration of Helsinki. This study was part of a larger research project focused on BFR-training. Other data from this project are reported elsewhere.

2.2. Diet

Participants consumed a standardised dinner and breakfast of own choice between 20 and 15 h and 2–3 h, respectively, before the first experimental day. These meals were replicated prior to the experimental day after the training period. In addition, the participants recorded their entire intake of food and beverages within two days prior to the first experimental day and were strictly instructed to copy this diet prior to the second experimental day. Participants were also instructed to maintain their routine diet throughout the study.

2.3. Experimental design

This study used a within-subject design. Each of the participants' legs was randomly allocated, in a counterbalanced order, to one of two six-week interventions consisting of interval cycling with (BFR-leg) or without (CON-leg) blood flow restriction, respectively. Randomisation was based on knowledge about the dominant (preferred kicking) leg. The legs were trained simultaneously and the work produced by the legs was matched during training sessions using real-time, visual feedback, as previously described [39]. Before the intervention and at least 72 h after the last training session, the participants completed an incremental exercise test to exhaustion in a Krogh ergometer, ensuring isolated exercise with the knee-extensor muscle group [42], to determine incremental leg peak power output (iPPO). This was followed 48–72 h later by an experimental day, on which glucose uptake in each leg was assessed during single-leg, isolated knee-extensor exercise at the same absolute, moderate, pre-training intensity (Ex1; 25% iPPO) and at the same relative, high intensity (Ex2; 90% iPPO) under intravenous infusion of first placebo (saline) followed by *N*-acetylcysteine. Thigh glucose uptake was assessed by measuring femoral arterial blood flow and femoral arterial and venous blood glucose concentrations. On the same day, a resting sample was collected from the vastus lateralis muscle of each leg to determine the protein abundance of endogenous antioxidant enzymes (CuZn-SOD, Mn-SOD, GPX-1, and catalase), GLUT4, nNOS, VEGF, AMPK and its downstream target, ACC, and the phosphorylation of AMPK at Thr^{172} (p-AMPK Thr^{172}) and ACC at Ser^{79}. The maximal oxygen uptake (VO_{2max}) and individual, relative training intensity was determined every second week during the intervention by performing an incremental cycling test to exhaustion on a bike ergometer (Monark Exercise, AB, Vansbro, Sweden). Experimental procedures were performed under standard laboratory conditions (~23 °C and ~33% humidity) at the August Krogh Building, Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen, Denmark. Participants were not informed about the study hypotheses to minimise a possible placebo effect. Further, they were instructed to avoid caffeine and alcohol and not to engage in vigorous physical activity in the 24 h leading up to each laboratory visit. They were also asked to keep a record of their

food and liquid intake in this 24-h period and asked to replicate the same diet prior to each visit.

2.4. Incremental knee-extensor exercise test

Upon arrival on this day, the participants warmed up at a fixed, absolute workload (5 min, 100 W, 75 rpm) on the same bike ergometer as used for the incremental cycling test (Monark Exercise, AB, Vansbro, Sweden), before being transferred to the Krogh ergometer. After 15 min of rest, the participants commenced the incremental knee-extensor exercise test with one leg (chosen at random before the training period), while seated upright in the ergometer with a hip angel of 120 degrees. The participants started the test by exercising for 5 min at 24 W, followed by a gradual increase in workload of 6 W per minute until exhaustion. The test was terminated when the required workload was not sustained at a kicking frequency ≥60 rpm. After at least 30 min of rest, the other leg was tested following the same procedure. The order of the legs was the same after as before the intervention. Participants were instructed to consume the same self-chosen, light meal 3 h before each test, as described in detail elsewhere [39]. During the test, only water was allowed on an ad libitum basis. The iPPO was calculated as the sum of the power output at the last completed stage and the product of the time spend at the last (incomplete) stage and the increment (6 W). An exercise intensity eliciting task failure after 3 to 9 min was established based on the first execution of the test. This intensity was used to determine the workload during the exercise bouts (Ex1 and Ex2) on the experimental day.

2.5. Incremental bike ergometer test

Upon arrival at the laboratory, participants were instrumented with cycling shoes with cleats and a facemask covering their mouth and nose. The facemask was connected by a slim tube to an online gas-analysing system (Oxycon Pro, Jaeger, Germany) for measurement of inspired and expired gases. The participants commenced the test by cycling for 4 min at 90 W, after which the workload was increased by 30 W every fourth minute until volitional fatigue, defined as a failure to withstand the required workload. Five min after reaching exhaustion, the participants performed another exercise period commencing at a workload corresponding to that of the last completed exercise stage, following by an increase in workload of 10 W per minute until exhaustion. The last exercise period was implemented to improve the estimation of VO_{2max}, which was calculated as the maximum 15-s plateau in oxygen uptake during the test. The maximal workload (W_{max}) used to estimate training intensity was calculated as the sum of the workload of the last completed stage and the product of the increment (30 W) and the fraction of time spend during the last (incomplete) stage.

2.6. Training

Training was performed on modern bike ergometers that ensured feedback in real-time about the workload and cadence (Tomahawk IC7, Cycling Group, Nürnberg, Germany). Training sessions were completed at a local gym (fitnessdk, Copenhagen, Denmark). Prior to each training session, each participant was instrumented with cycling shoes with force-sensor insoles. The insoles were wirelessly connected to a display placed in front of the participants, allowing visual feedback in real-time about the work produced by each leg during every pedal rotation, according to the procedure recently described [39]. No difference was recorded in the work produced by CON-leg and BFR-leg during the intervention ($p > 0.05$). A training session consisted of a warm-up (5 min at 30% W_{max}) and two minutes of rest, followed by nine two-minute cycling bouts. The bouts were performed in series of three, with bouts and series separated by 1 and 5 min, respectively, of active recovery (pedalling freely without resistance). The target intensity in the first, second and third series was 60%, 70% and 80% of W_{max}, correspondingly. Participants cycled at a fixed cadence at their choice, which ranged from 75 to 85 rpm. Before the intervention, the participants were familiarised to the training protocol and equipment for four weeks incorporating biweekly training sessions, with sessions interspersed by at least 48 h. During the familiarisation, the participants performed 8 ± 3 (mean ± SD) sessions. During the intervention, they completed 17 ± 1 (mean ± SD) sessions, which corresponded to three weekly sessions. Rate of perceived exertion (as assessed using the 6–20 BORG-scale) during the intervention was on average 13 ± 1 in the first, 16 ± 2 in the second, and 19 ± 1 in the third series. The recorded training intensity was 61 ± 3, 72 ± 4, and 81 ± 10% W_{max} for the same periods. Verbal encouragement was provided by at least one investigator during training sessions.

2.7. Blood flow restriction

In the BFR-leg, thigh blood flow was reduced during exercise bouts by inflation of a nylon cuff (13 cm in width; Riester, Germany) around the proximal portion of the thigh to an external pressure of ~180 mmHg, in accordance with the method previously described [18]. This cuff pressure reduces thigh blood flow by approximately 50% during exercise and increases muscle blood flow in the subsequent recovery by >3 fold [37].

2.8. Experimental day

Two hours after consuming a standardised breakfast, the participants arrived at the research facility at 8 am in the morning to undertake the main experiment. Upon arrival, participants were placed on a hospital bed, where catheters were inserted into the femoral artery and vein of both legs under local anaesthesia (20 mg/mL lidocaine). After a period of rest, the participants commenced the single-leg, knee-extensor exercise protocol in the same Krogh ergometer as used during the familiarisation and prior testing. The experimental protocol is shown in Fig. 1. The protocol consisted of 10 min of exercise at 25% iPPO (Ex1), followed 25 min later by exercising at 90% iPPO to exhaustion (Ex2), defined as a failure to maintain exercising at the required workload at ≥50 rpm. If participants continued exercising beyond 4 min in Ex2, the intensity was raised by 6 W every minute until exhaustion. After completion of the protocol with one leg, the contralateral leg commenced the same protocol after approximately 45 min of rest. In the fifteen min before and during the first completion of Ex1 and Ex2 with both legs, placebo (saline; 20 mg/mL) was intravenously injected at a constant rate of 10 mL/h. After another ~45 min of rest, the protocol was repeated with both legs in the same order under the infusion of *N*-acetylcysteine (NAC). The fixed order of infusions on experimental days was chosen, because the reduced form of NAC in blood has a half-life of almost 7 h [43], whereas a doubling of the invasive, experimental days would be unsuitable to complete for participants (i.e., unethical). Twenty min before the start of the second round of exercise with each leg, NAC was infused for 15 min at a rate of 125 mg/kg/h, after which the rate was reduced to and maintained at 25 mg/kg/h for the remainder of the experiment, in line with previous procedure [34]. This NAC infusion protocol increases skeletal muscle cysteine and glutathione levels in humans [34]. Participants were blinded and did not report any negative effects of any infusion. During NAC injection, Ex2 was terminated at the point of task failure reached during exercise with placebo. Participants were instructed to keep a cadence of 60 rpm at all time while exercising. Blood samples were drawn from the exercising thigh before and after the start of infusions while seated, and after 20 s, and 1, 3, and 8 min of Ex1, after 20 s, 1, and 3 min of Ex2, and 20 s, and 1, 2, and 3 min in the recovery from each exercise bout. The blood mean transit time from the arterial to venous site was accounted for by collecting the venous sample five s after the arterial sample [44]. Femoral arterial blood flow of the exercising leg was measured continuously by ultrasound Doppler from five s before sampling of blood to venous sample

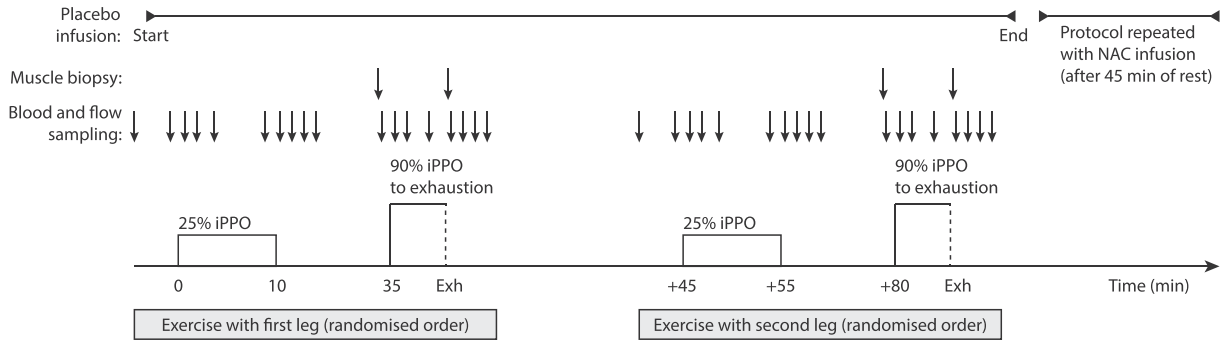

Fig. 1. Illustration of the experimental day. iPPO: incremental peak aerobic power output; NAC: *N*-acetylcysteine.

collection. A muscle biopsy was obtained approximately five min before the first execution of, as well as immediately after, Ex2. Only the resting biopsy prior to Ex2 was used for analysis in the present study. The workload was the same for placebo and NAC infusions. The mean (± SD) workload in Ex1 for CON-leg and BFR-leg was 17 (± 4) and 16 (± 4) W, respectively, before training. After training, the corresponding values were 18 (± 3) and 20 (± 3) W. The mean (± SD) duration of Ex2 for CON-leg and BFR-leg was 4:52 (± 1:35) and 4:44 (± 1:07) min with placebo, and 4:37 (± 1:30) and 4:29 (± 1:16) min with NAC infusion before training. After training, the corresponding values were 4:50 (± 1:34) and 4:43 (± 1:07) with placebo and 4:37 (± 1:29) and 4:29 (± 1:15) min with NAC infusion, respectively. These values were first reported elsewhere [39].

2.9. Measurement of thigh blood flow

Blood flow in the femoral artery was measured noninvasively by ultrasound Doppler (Logic E9, GE Healthcare, Pittsburg, PA, USA). Image and Doppler frequency was 9 and 3.1 MHz, respectively. Blood velocity was assessed distal to the inguinal ligament above the bifurcation into the superficial and profound arterial branch. Insonation angle during measurements was <60 degrees and kept as low as possible. Vessel lumen without walls was considered as sample volume. Signal noise originating from turbulence near the vascular walls was removed by application of a low-velocity filter (<1.8 m/s). Doppler tracings and B-mode images were recorded continuously. A mean of tracings was recorded during blood sampling and used for final analysis. Vessel diameter was estimated for each measurement and artery diameter was determined during systole at rest and at maximal vessel expansion during exercise and subsequent recovery. Artery diameter was recorded as the mean of three measurements perpendicular to the vessel wall at the site where blood velocity was measured.

2.10. Blood sampling

Approximately 2 mL of blood was collected using a single-use, heparinised syringe. Samples were instantaneously stored on ice, followed by analysis for glucose concentration on an ABL 800 Flex (Radiometer, Copenhagen, Denmark). Prior to sample collection, approximately 2 mL of blood was drawn to ensure sampling of circulating blood, with the blood being reintroduced into the venous circulation after the recovery.

2.11. Muscle sampling

Muscle was sampled from the vastus lateralis muscle using the Bergström needle biopsy technique with suction. Prior to sampling, a scalpel blade was used to make an incision (approximately 1 cm in width) through the skin and muscle fascia under local anaesthesia (2–3 mL of 1% Xylocaine). Samples were immediately frozen in liquid nitrogen and stored at −80 °C until subsequent analysis. At the end of the day, the incisions were cleaned with ethanol and covered with sterile Band-Aid strips and a Tegaderm film-dressing.

2.12. Muscle sample preparation for immunoblotting

Approximately 15 mg w.w. muscle per sample was freeze-dried for 48 h. Freeze-dried samples were dissected free of connective tissue, fat, and blood under a stereo microscope using jeweller's forceps under standard environmental conditions (22 °C, <30% humidity). Dissected samples were homogenised (two periods of 30 s at 28.5 Hz) in a fresh batch of ice-cold MG-buffer (10% glycerol, 20 mM Na^+-pyrophosphate, 150 mM NaCl, 50 mM HEPES with a pH of 7.5, 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, 2 mM PMSF, 1 mM EDTA with a pH of 8, 1 mM EGTA with a pH of 8, 10 μg/mL

Table 1
Primary antibodies used for immunoblotting.

Protein	Primary antibody and supplier	Host species and isotype (antibody type)	Concentration	Molecular mass (kDa)
nNOS	BD Transduction Laboratories (#610309)	Mouse, IgG (monoclonal)	1:5.000	~150
GLUT4	Thermo Fisher Scientific (#PA1-1065)	Rabbit, IgG (polyclonal)	1:5.000	~50
VEGF	Santa Cruz Biotechnology Inc. (#sc-152)	Rabbit, IgG (polyclonal)	1:250	~20 and ~40
p-AMPK Thr^{172}	Cell Signaling (#2531s)	Rabbit, IgG (polyclonal)	1:1.000	~65
AMPK	Cell Signaling (#2532s)	Rabbit, IgG (polyclonal)	1:1.000	~65
p-ACC Ser^{79}	Merck Millipore (#07-303)	Rabbit, IgG (polyclonal)	1:1.000	~250
ACC	Cell Signaling (#3676)	Rabbit IgG (monoclonal)	1:1.000	~250
Cu/Zn-SOD	Cell Signaling (#2770)	Rabbit IgG (polyclonal)	1:1.000	~18
Mn-SOD	Merck Millipore (#06-984)	Rabbit IgG (polyclonal)	1:5.000	~24
GPX-1	Abcam (#ab22604)	Rabbit IgG (polyclonal)	1:1.000	~22
Catalase	Abcam (#ab1877)	Rabbit IgG (polyclonal)	1:5.000	~60

GLUT4 and VEGF were diluted in 2 and 5% milk, respectively. All other antibodies were diluted in 3% bovine serum albumin in Tris-buffered saline with 0.025% Tween.

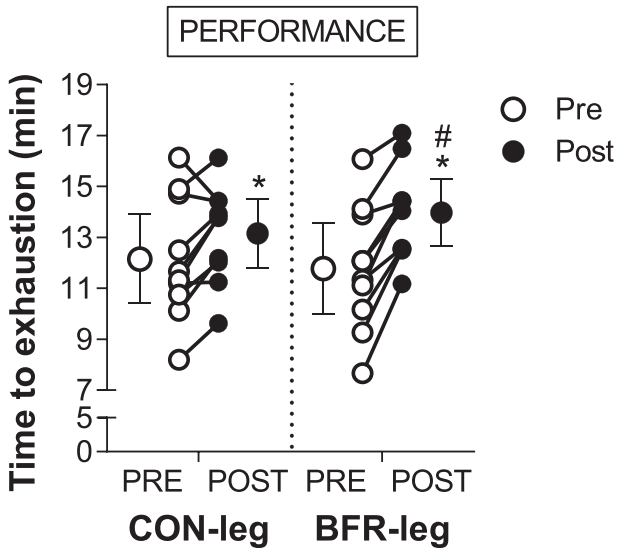

Fig. 2. Performance (time to exhaustion) during exhaustive exercise before (Pre; open circles) and after (Post; closed circles) training without (CON-leg) or with blood flow restriction (BFR-leg). Values are shown as means ± 95% confidence intervals. Individual changes are also shown (connecting circles).

Aprotinin, 10 μg/mL Leupeptin and 3 mM Benzamidine) using a TissueLyser (Qiagen TissueLyser II, Retsch GmbH, Haan, Germany). Samples were not fractionated; hence crude homogenates were used for subsequent analyses. Protein concentration in samples was determined using a BSA protein assay kit (Thermo Scientific, USA). Based on protein concentration, homogenised samples were further diluted to a final concentration of 2 μg/uL by addition of denaturing buffer (7 mL 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue). Denatured samples were stored at −80 °C until immunoblotting.

2.13. Immunoblotting

Protein abundance and phosphorylation were determined by western blotting. For each sample, 10 μg of protein were loaded into an individual well on a 4–15% Criterion TGX stain-free gel (Bio-Rad, Hercules, CA). Samples from five subjects, a 4-point standard curve of crude human whole-muscle homogenate with a known protein concentration (2 μg/uL), and two protein size markers (Precision Plus Protein All Blue, #1610373, Bio-Rad), were loaded onto each gel. Electrophoresis was performed for 45 min at 200 V, followed by UV-activation of gels for 5 min using a ChemiDoc image station (Bio-Rad) to enable stain-free quantification of total protein. Protein was transferred (45 min at 14 V) onto 0.45 μm PVDF-membrane (Immobilon P, Millipore) using a semi-dry transfer station (Amersham TE 70, GE Healthcare) and transfer buffer (25 mM Tris-base, 190 mM glycine, 0.015% SDS, and 20% ethanol). Following transfer, membranes were blocked in 5% skimmilk by gently rocking for 1 h at room temperature. Membranes were cut horizontally at the desired molecular size using protein markers to allow multiple proteins to be quantified on the same membrane. Membranes were incubated with primary antibody overnight at 4 °C using gentle

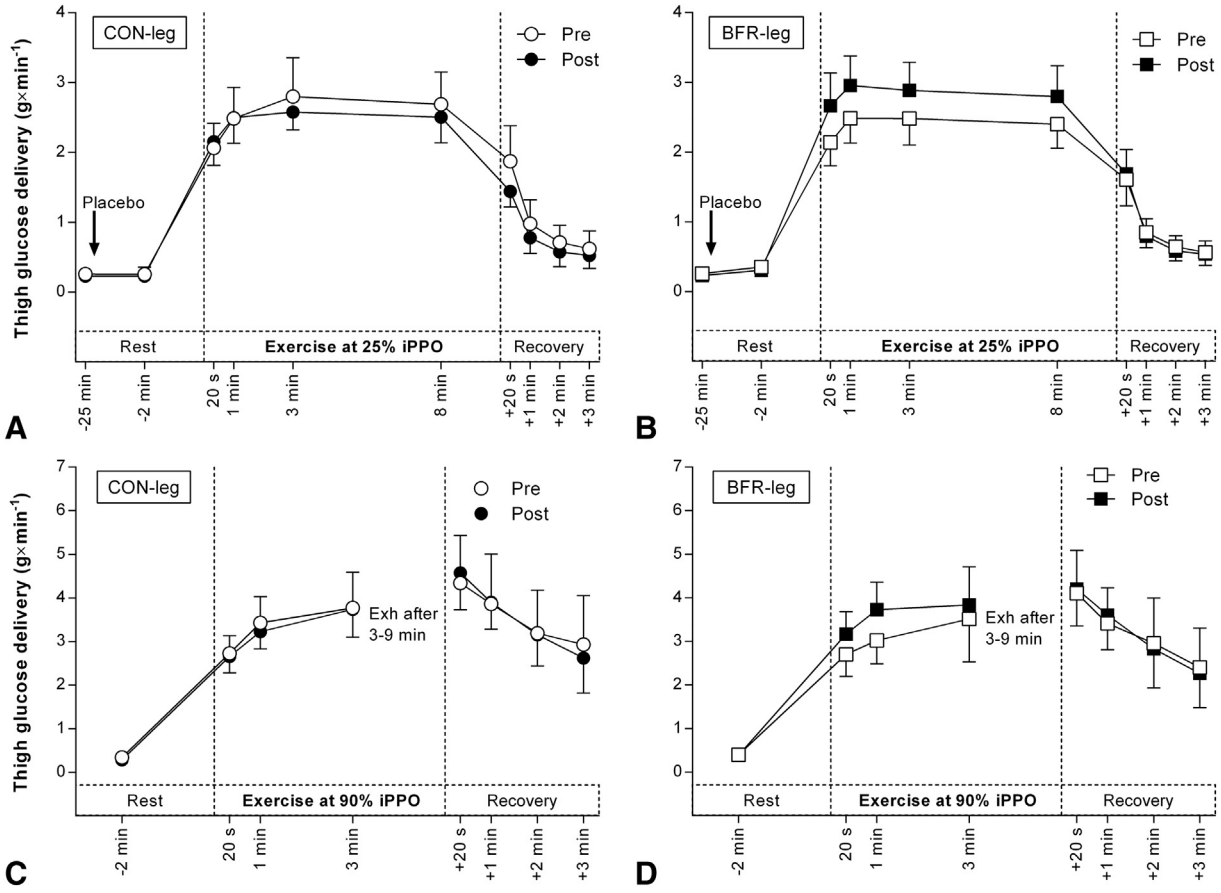

Fig. 3. Effect of training with and without blood flow restriction (BFR) on thigh glucose delivery. A) Thigh glucose delivery in the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Thigh glucose delivery in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Thigh glucose delivery in CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Thigh glucose delivery in BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals.

rocking. Antibody details are presented in Table 1. After incubation, membranes were washed several times in TBST, before being incubated by gently rocking in HRP-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualised by chemiluminescence using Immobilon Forte Western HRP Substrate (#WBLUF0500, Merck Millipore, USA) on a ChemiDoc MP imaging station (Bio-Rad). Protein quantification was completed in Image Lab 5.2.1 software (Bio-Rad). Sample protein abundance was determined by normalising sample density to that of the calibration curve slope and total protein in the sample lane on the stain-free gel image. Linearity of calibration curves was $r^2 = \geq 0.98$. In some cases, one point on each calibration curve was excluded from analysis, because the band was considered unreliable after visual inspection due to either noise on the image caused by artefacts or if it was too faint.

Calculations

Thigh glucose delivery was calculated as the product of thigh blood flow and femoral arterial glucose concentration. Thigh glucose extraction was determined as the difference between venous and arterial glucose concentration (v-a glucose difference). Net thigh glucose uptake was calculated as the product of femoral arterial blood flow and v-a glucose difference.

3. Statistics

Data are expressed as means ± 95% confidence intervals. Probability is presented as p-values using an alpha-level of $p \leq 0.05$ to indicate statistical significance. Data were assessed for normality using the Shapiro Wilks test. A two-way ANOVA with repeated measures was used to assess the effect of training on performance and between-leg differences for change in performance using time and leg as factors. A linear mixed model was used to determine within-leg changes and between-leg differences. For glucose delivery, extraction, and uptake, the model used leg, infusion, exercise intensity, and sample no. as fixed factors and a random intercept for subjects. For protein abundance and phosphorylation, leg and time were used as fixed factors and subjects as random factor. Data points were excluded from analysis if deviating >2 standard deviations from the mean. This resulted in removal of outliers for GLUT4 and catalase (n = 1 for each time point in both legs), VEGF and ACC (n = 1 at Post in BFR-leg), nNOS (n = 1 for each time point in CON-leg and n = 1 at Pre in BFR-leg), p-AMPK (n = 1 at Post in CON-leg), AMPK (n = 1 at Pre in BFR-leg), p-AMPK/AMPK (n = 1 at each time point in BFR-leg, n = 1 at Pre in CON-leg), p-ACC (n = 1 at Pre in CON-leg), and Mn-SOD (n = 1 at Pre in BFR-leg, n = 1 at Post in CON-leg). *Posthoc* comparisons used the Tukey's test. Effect size (d) used Cohen's conventions, where <0.2, 0.2–0.5, >0.5–0.8, and>0.8 was considered a trivial, small, moderate and large effect,

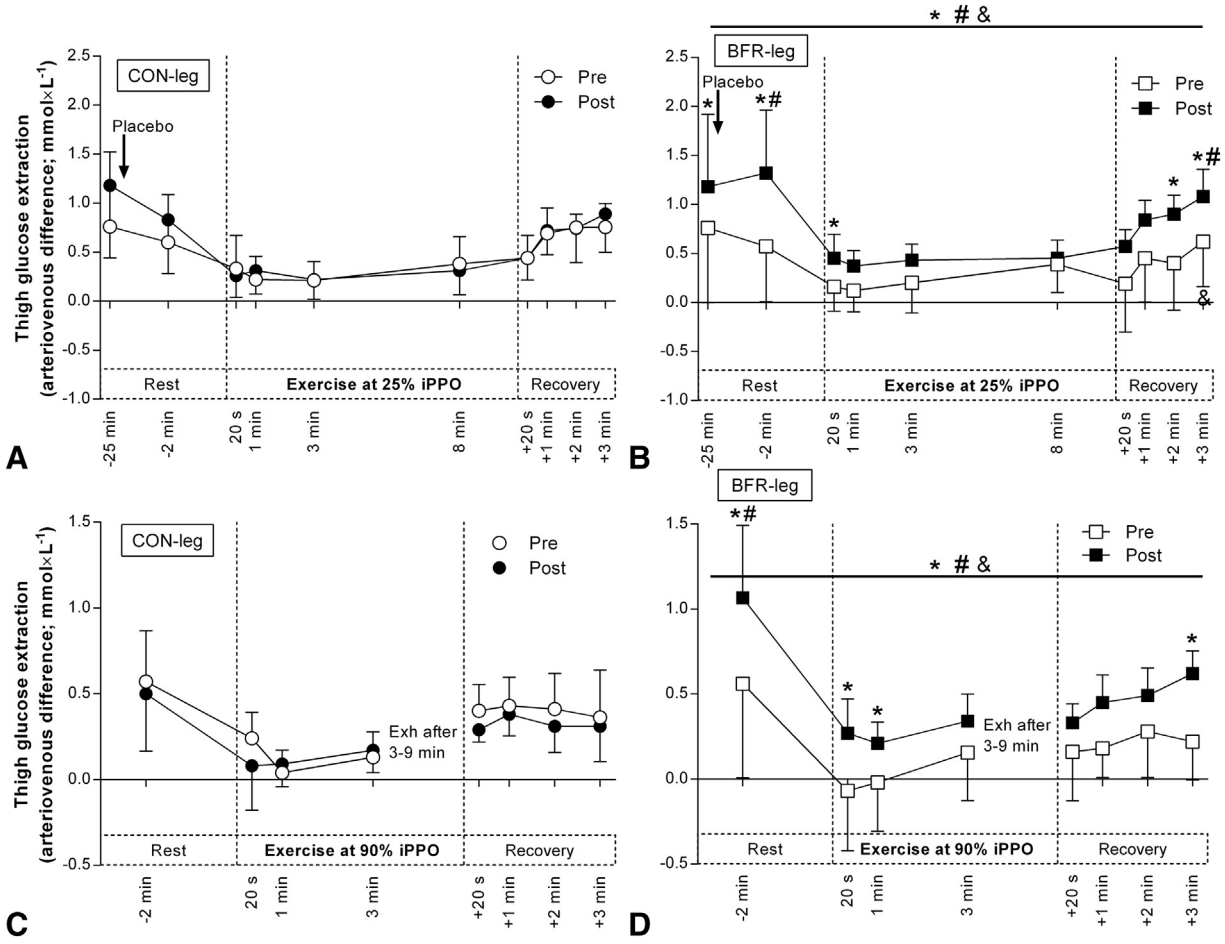

Fig. 4. Effect of training with and without blood flow restriction (BFR) on thigh glucose extraction. A) Thigh glucose extraction in the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Thigh glucose extraction in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Thigh glucose extraction in CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Thigh glucose extraction in BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals. *p < 0.05, higher Post than Pre in BFR-leg; #p < 0.05, higher than CON-leg at Post; &p < 0.05, lower than CON-leg at Pre.

respectively [45]. Statistical analyses were performed in SPSS version 17 and Sigma Plot version 11.

4. Results

4.1. Performance

Before training, time to exhaustion was not different between legs ($p = 0.1$). There was a significant time effect ($p = 0.001$) and leg x time interaction ($p = 0.001$) for time to exhaustion. Time to exhaustion increased with training in CON-leg ($10 \pm 7\%$; $p = 0.036$) and in BFR-leg ($21 \pm 8\%$; $p < 0.001$), with the increase being greater in BFR-leg than in CON-leg ($11 \pm 5\%$; $p = 0.001$; Fig. 2).

4.2. Thigh glucose uptake

Training had no effect on thigh glucose delivery in either leg at both exercise intensities ($p = 0.25$). No leg x time interaction was found for glucose delivery ($p = 0.11$), and no differences were observed between legs either before ($p \geq 0.48$) or after ($p \geq 0.47$) training (Fig. 3).

In BFR-leg, thigh glucose extraction increased with training at both exercise intensities ($p \leq 0.001$). In contrast, in CON-leg, thigh glucose extraction remained unaltered by training ($p \geq 0.10$). A significant leg x time interaction was found for glucose extraction ($p < 0.001$). Before training, thigh glucose extraction was lower in BFR-leg than in CON-leg at both intensities ($p \leq 0.03$). After training, thigh glucose extraction was higher in BFR-leg than in CON-leg at both intensities ($p \leq 0.03$) (Fig. 4).

In BFR-leg, thigh net glucose uptake increased with training at both exercise intensities ($p < 0.001$), whereas in CON-leg, thigh net glucose uptake remained unaltered by training at both intensities ($p \geq 0.23$). A significant leg x time interaction was found for glucose uptake ($p < 0.001$). Before training, thigh net glucose uptake was lower in BFR-leg than in CON-leg during exercise at 25% iPPO ($p = 0.61$), but not at 90% iPPO ($p = 0.29$). After training, thigh net glucose uptake was higher in BFR-leg than in CON-leg at both intensities ($p \leq 0.01$) (Fig. 5).

4.3. GLUT4, nNOS, and VEGF abundance

In BFR-leg, GLUT4 abundance was ~28% higher after training ($p = 0.026$; $d = 0.8$), whereas it was unchanged by training in CON-leg (~8%; $p = 0.58$; $d = 0.2$). GLUT4 abundance was not different between legs either before or after training ($p = 0.77$; $d = 0.4$ at Pre, $d = 0.3$ at Post) (Fig. 6A). In BFR-leg, nNOS abundance was ~18% higher after training ($p = 0.029$; $d = 0.4$), whereas it was unchanged by training in CON-leg (~11%; $p = 0.28$; $d = 0.3$). nNOS abundance was not different between legs either before or after training ($p = 0.97$; $d = 0.3$ at Pre, $d = 0.2$ at Post) (Fig. 6B). In both legs, VEGF abundance remained unaltered by training (mean increase of ~20 and ~21% in CON-leg and BFR-leg, respectively), irrespective of whether all or individual VEGF splice variants were quantified ($p \geq 0.5$; $d = 0.5$ for CON-leg, $d = 0.1$ for BFR-leg). VEGF abundance was not different between legs either before

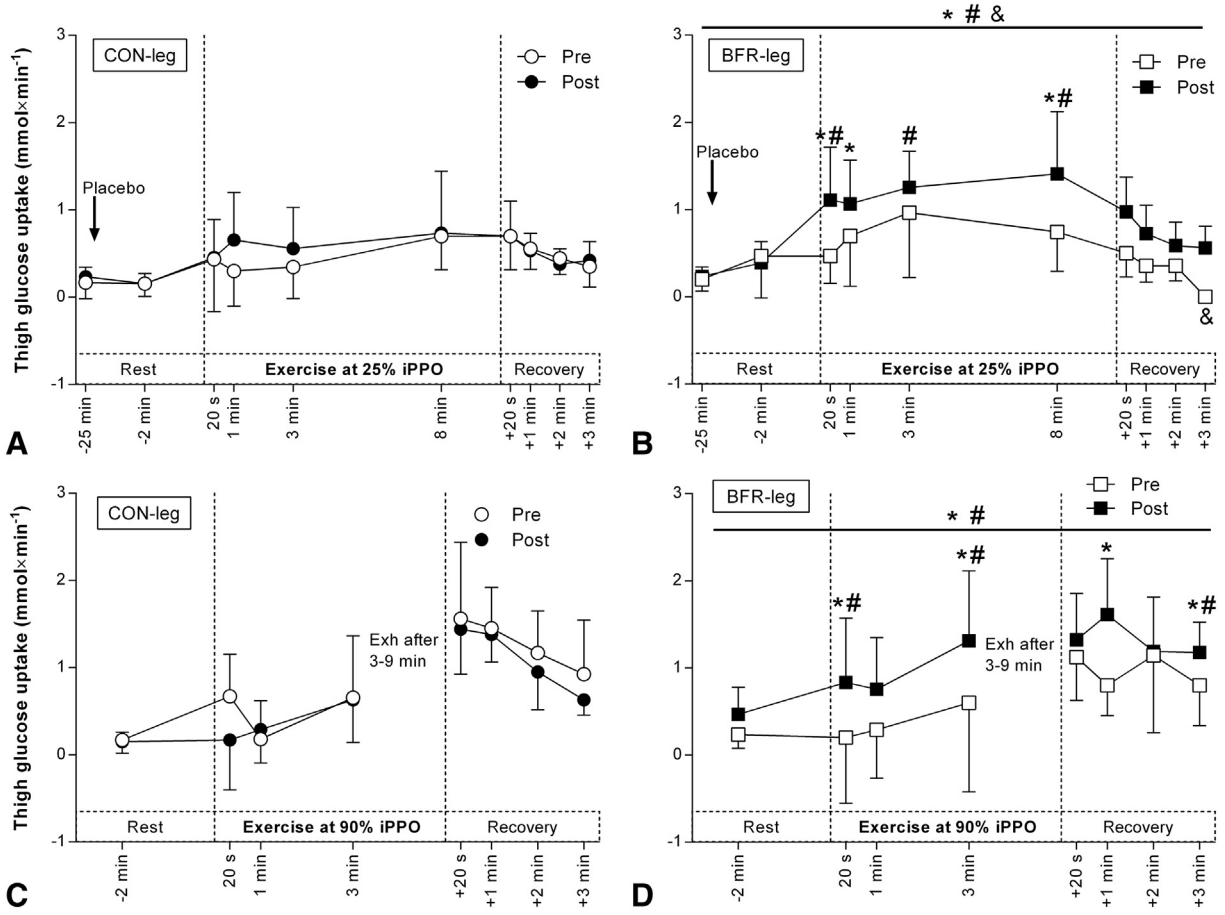

Fig. 5. Effect of training with and without blood flow restriction (BFR) on net thigh glucose uptake. A) Net thigh glucose uptake in the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Net thigh glucose uptake in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Net thigh glucose uptake in CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Net thigh glucose uptake in BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals. *p < 0.05, higher Post than Pre in BFR-leg; #p < 0.05, higher than CON-leg at Post; &p < 0.05, lower than CON-leg at Pre.

or after training irrespective of the number of splice variants quantified ($p \geq 0.2$; $d = 0.1$ at Pre, $d = 0.6$ at Post) (Fig. 6C).

4.4. AMPK and ACC abundance and phosphorylation

In BFR-leg, p-AMPK Thr^{172} was ~38% higher after training ($p = 0.018$; $d = 0.9$), whereas it was unchanged by training in CON-leg (~51%, $p = 0.15$; $d = 0.7$). p-AMPK Thr^{172} was not different between legs either before or after training ($p \geq 0.08$; $d = 0.4$ at Pre, $d = 0.7$ at Post) (Fig. 7A). In both legs, AMPK abundance was unchanged by training (CON-leg: ~18, $d = 0.4$; BFR-leg: ~16%, $d = 0.2$; $p = 0.2$), with no difference between legs either before or after training ($p = 0.2$; $d = 0.1$ at Pre and Post) (Fig. 7B). Similarly, in both legs, the p-AMPKThr^{172}/AMPK ratio was unchanged by training (CON-leg: ~21, $d = 0.7$; BFR-leg: ~22%, $d = 0.1$; $p = 0.1$), with no difference between legs either before or after training ($p = 0.6$; $d = 0.5$ at Pre, $d = 0.2$ at Post) (Fig. 7C).

In both legs, p-ACC Ser^{79} remained unaltered by training (CON-leg: ~34, $d = 0.4$; BFR-leg: ~13%, $d = 0.2$; $p \geq 0.5$), with no difference between legs either before or after training ($p \geq 0.1$, $d = 0.1$ at Pre, $d = 0.4$ at Post) (Fig. 7D). In both legs, ACC abundance was also unchanged by training ($p = 0.1$, $d = 0.1$ for CON-leg, $d = 0.2$ for BFR-leg). ACC abundance was not different between legs either before or after training ($p = 0.2$, $d = 0.1$ at Pre, $d = 0.2$ at Post) (Fig. 7E). In both legs, the p-ACC Ser^{79}/ACC ratio was unchanged by training (CON-leg: ~43, $d = 0.1$; BFR-leg: ~13%, $d = 0.1$; $p = 0.1$). The p-ACC Ser^{79}/ACC ratio was higher in

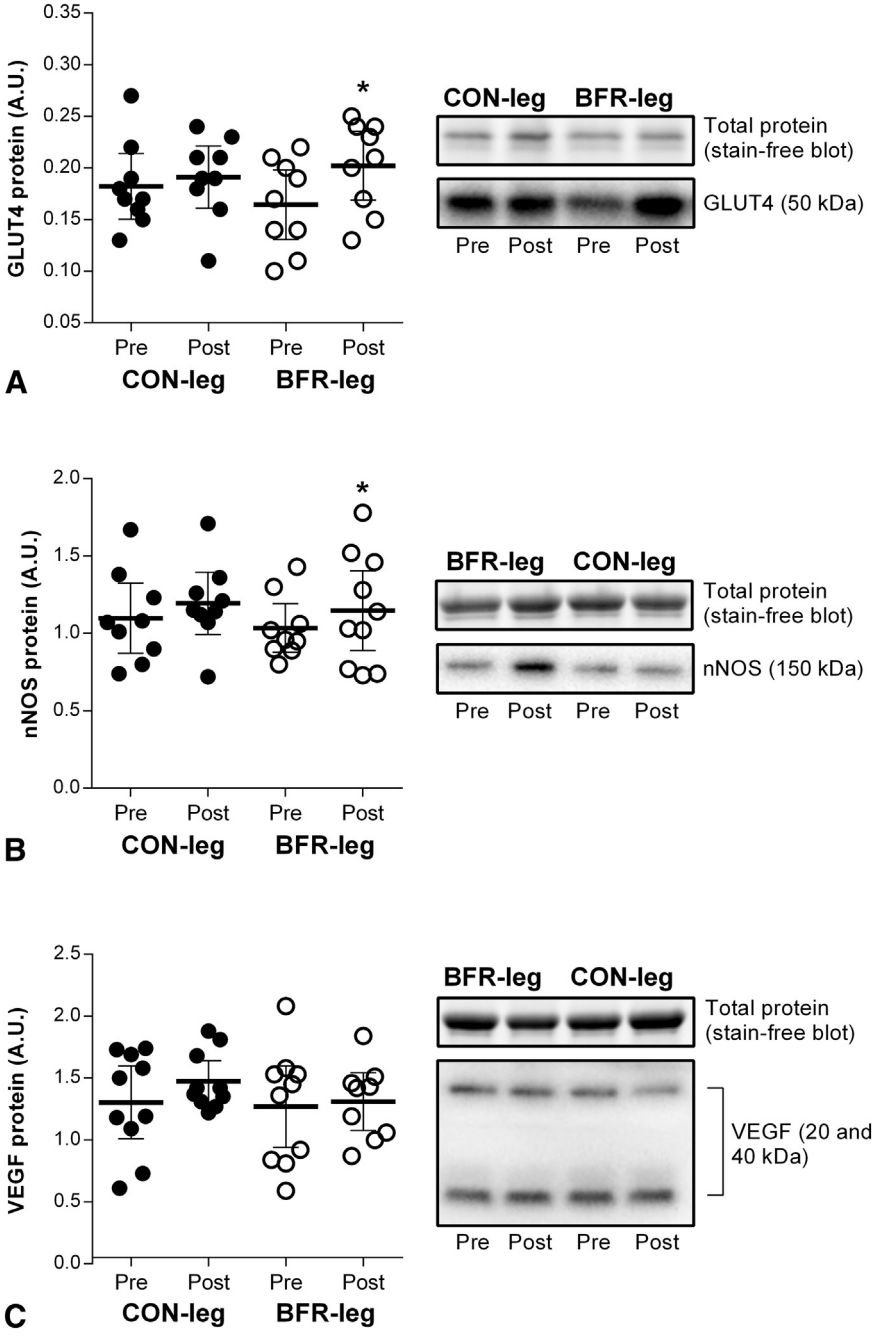

Fig. 6. Effect of six weeks of training without (CON-leg) or with blood flow restriction (BFR-leg) on A) GLUT4, B) nNOS, and C) VEGF abundance in human muscle. Values before (Pre) and after (Post) the training period are expressed as means ± 95% confidence intervals and individual values are shown either as solid circles for CON-leg or open circles for BFR-leg. *$p < 0.05$, higher Post than Pre in BFR-leg. Representative western blots of the proteins and total protein loaded onto the stain-free gels are shown next to the respective protein figures.

CON-leg than in BFR-leg after training (~31%; p = 0.01; d = 0.5), but no difference was observed between legs before training (p = 0.1; d = 0.3) (Fig. 7F).

4.5. Effect of antioxidant infusion on thigh glucose uptake

Before training, NAC increased thigh glucose delivery in BFR-leg during exercise at 90% iPPO (p = 0.01; vs. placebo), but did neither affect thigh glucose delivery at 25% iPPO in the same leg (p = 0.09; vs. placebo) nor in CON-leg at either intensity (p ≥ 0.10; vs. placebo). After training, NAC did not affect thigh glucose delivery in either leg at both exercise intensities (p ≥ 0.29; vs. placebo). No leg x time x infusion interaction was found for glucose delivery (p = 0.21). Accordingly, the effect of NAC on thigh glucose delivery was not different between legs either before (p ≥ 0.33) or after training (p = 0.55) (Fig. 8).

Before training, NAC had no effect on thigh glucose extraction in BFR-leg at both exercise intensities (p ≥ 0.23; vs. placebo). Similarly, in CON-leg, NAC was without effect thigh glucose extraction (p ≥ 0.45; vs. placebo). After training, NAC decreased thigh glucose extraction in BFR-leg at both intensities (p ≤ 0.004; vs. placebo). In contrast, NAC had no effect on thigh glucose extraction in CON-leg after training (p ≥ 0.13). A significant leg x time x infusion interaction was found for glucose extraction (p = 0.057). Before training, the effect of NAC was not different between legs (p ≥ 0.32). After training, the effect of NAC was greater in BFR-leg than in CON-leg at both intensities (p ≤ 0.004) (Fig. 9).

Before training, NAC increased thigh net glucose uptake in BFR-leg during exercise at both 25% iPPO (p = 0.051; vs. placebo) and 90% iPPO (p = 0.001; vs. placebo), but did not affect thigh net glucose uptake in CON-leg at either intensity (p ≥ 0.78; vs. placebo). After training, NAC decreased thigh net glucose uptake in BFR-leg at both intensities (p ≤ 0.047; vs. placebo). In contrast, NAC had no effect on thigh net glucose uptake in CON-leg at either intensity after training (p ≥ 0.60; vs. placebo). A significant leg x time x infusion interaction was found for glucose uptake (p < 0.001). However, the effect of NAC was not different between legs at either intensity before (p ≥ 0.46) or after training (p ≥ 0.16) (Fig. 10).

4.6. Antioxidant enzyme abundance

In BFR-leg, Cu/Zn-SOD abundance was ~39% higher after training (p = 0.021; d = 1.0). In contrast, Cu/Zn-SOD abundance was unchanged by training in CON-leg (~13%; p = 0.6; d = 0.3). Cu/Zn-SOD abundance was not different between legs either before or after training (p = 0.2; d = 0.1 at Pre, d = 0.6 at Post) (Fig. 11A). In both legs, Mn-SOD abundance remained unaltered by training (CON-leg: ~1%, d = 0.3; BFR-leg: ~52%, d = 0.9; p = 0.2). Mn-SOD abundance was higher in BFR-leg than in CON-leg after training (p = 0.041, d = 0.8), but not before (p = 0.4, d = 0.4) (Fig. 11B). In BFR-leg, GPX-1 abundance was ~29%

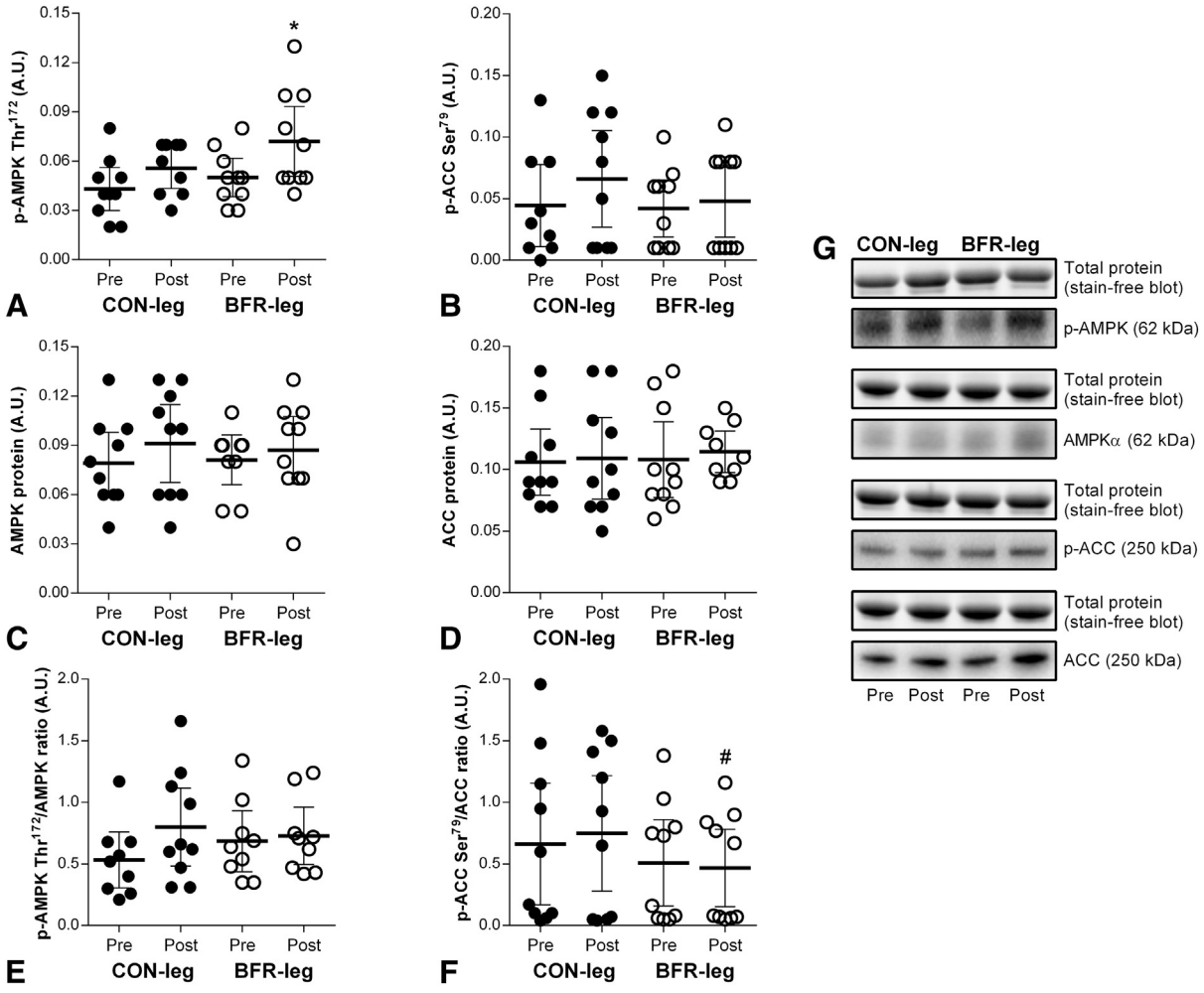

Fig. 7. Effect of six weeks of training without (CON-leg) or with blood flow restriction (BFR-leg) on A) p-AMPK Thr^{172}, B) p-ACC Ser^{79}, C) AMPK, and D) ACC abundance, and on E) p-AMPK Thr^{172}/AMPK and F) p-ACC Ser^{79}/ACC ratios in human muscle. Values before (Pre) and after (Post) the training period are expressed as means ± 95% confidence intervals and individual values are shown either as solid circles for CON-leg or open circles for BFR-leg. *p < 0.05, higher Post than Pre in the respective leg. #p < 0.05, lower in BFR-leg than in CON-leg at Post. G) Representative western blots of the proteins and total protein loaded onto the stain-free gels.

higher after training ($p = 0.001$; $d = 0.9$). In contrast, GPX-1 abundance was unchanged by training in CON-leg (~15%; $p = 0.1$; $d = 0.7$). GPX-1 abundance was not different between legs either before or after training ($p = 0.2$; $d = 0.0$ at Pre, $d = 0.5$ at Post) (Fig. 11C). In both legs, catalase abundance remained unaltered by training (CON-leg: ~24, $d = 0.4$; BFR-leg: 6%, $d = 0.4$; $p = 0.8$), with no differences between legs either before or after training ($p = 0.15$; $d = 0.9$ at Pre, $d = 0.3$ at Post) (Fig. 11D).

5. Discussion

The key novel findings of the present study were that blood-flow-restricted (BFR) training, in contrast to work-matched training without BFR, markedly enhanced thigh net glucose uptake during exercise in men. This was temporally associated with an improved endurance performance (i.e., time to exhaustion during exhaustive exercise), compared to the leg training without BFR. The greater net glucose uptake in the BFR-trained leg was caused by an increase in thigh glucose extraction and was associated with an elevated muscle GLUT4 abundance. Furthermore, intravenous infusion of antioxidant (*N*-acetylcysteine; NAC) attenuated thigh net glucose uptake during exercise only in the BFR-trained leg, which coincided with an elevated muscle abundance of nNOS and the antioxidant enzymes Cu/Zn-SOD and GPX-1, without changes in Mn-SOD and catalase abundance. In addition, resting muscle AMPK phosphorylation at Thr^{172} increased only in the BFR-trained leg, whereas the p-AMPK Thr172/AMPK ratio remained unchanged with training irrespective of the use of BFR.

5.1. Enhancement of thigh glucose uptake by training with BFR

The present study showed that only the leg that trained with BFR elicited increases in thigh net glucose uptake during exercise. Other human studies have documented the effectiveness of exercise training to increase thigh net glucose uptake at rest (ice hockey/bandy) [46], during exercise at the same high relative intensity (3 weeks with 3–4 sessions of 1–2 h of knee-extensor exercise at 70–85% of peak workload) [6], and at a given insulin dose (10 weeks with 6 sessions of 30 min of single-leg cycling at 70% of leg VO_{2max}) [4]. However, in the present study, training without BFR did not affect glucose uptake. This might have been due to the low relative training intensity in the CON-leg compared to the participants' routine physical activity. Given our experimental design, where one leg trained with and the other leg without BFR, local (i.e., muscular) rather than blood-borne factors must have caused the increase in thigh glucose uptake by BFR-training. In agreement, BFR increases muscle lactate accumulation, oxidative stress [18], and perturbations in oxygen perfusion [39] during interval exercise in recreationally-active men. These findings indicate that high muscle metabolic and/or redox stress during training sessions is fundamental for training-induced increases in thigh glucose uptake by exercising muscles in humans. Accordingly, BFR-training raised the abundance of

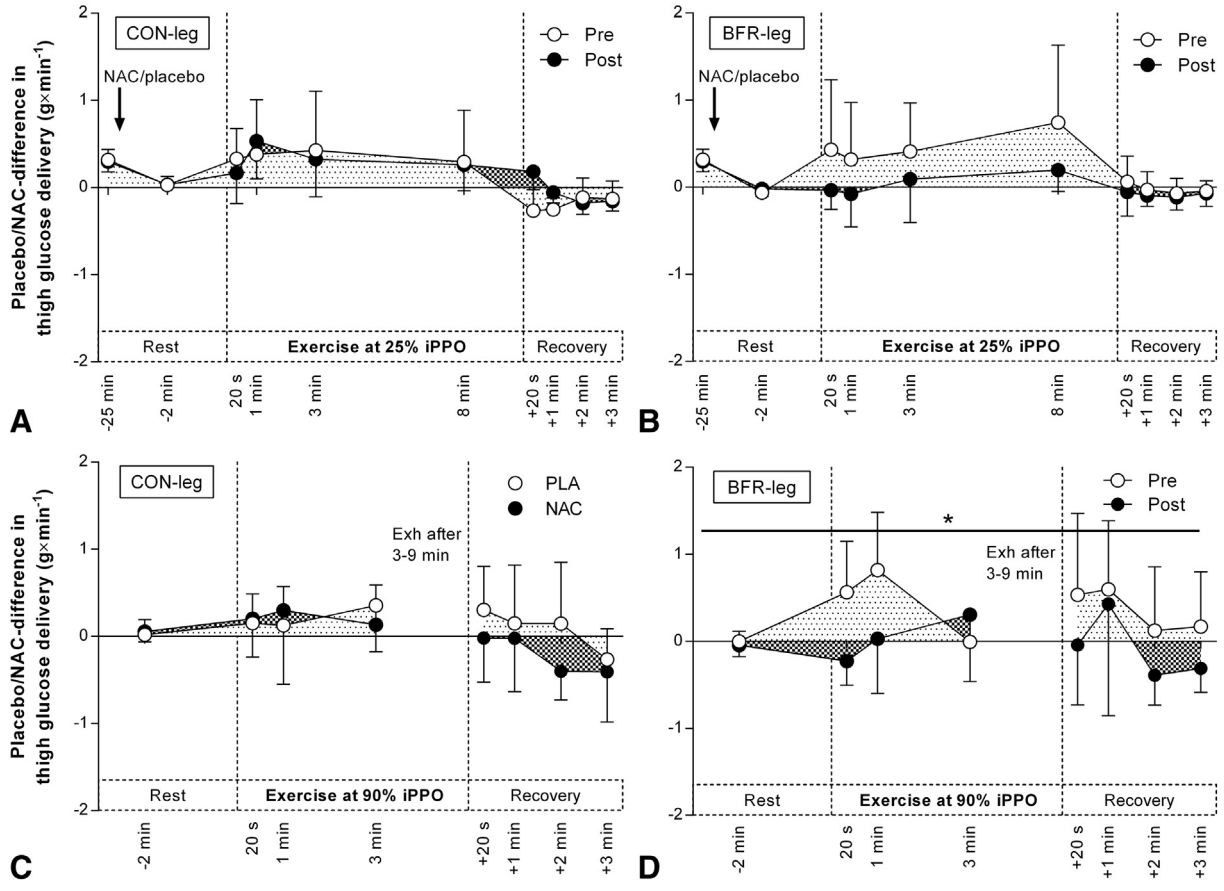

Fig. 8. Effect of *N*-acetylcysteine (NAC) infusion on thigh glucose delivery before and after training. A) Difference in thigh glucose delivery between NAC and placebo for the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Difference in thigh glucose delivery in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Difference in thigh glucose delivery between NAC and placebo for CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Difference in thigh glucose delivery between NAC and placebo for BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals. *$p < 0.05$, NAC higher than placebo at Pre.

some antioxidant enzymes (Cu/Zn-SOD and GPX-1) in skeletal muscle (Fig. 11), an effect that has been linked to the accumulation of ROS [47].

5.2. Mechanisms underlying enhancement of thigh glucose uptake by BFR-training

The enhanced thigh glucose uptake after training with BFR was caused by an increase in thigh glucose extraction and not by modulation of glucose delivery (Figs. 3 and 4). This may have been associated with the selective increase in muscle GLUT4 abundance (~30%) in the BFR-trained leg, and thus an improved potential for GLUT4 translocation and transmembrane glucose diffusion [48]. In support, we found a positive correlation between GLUT4 abundance and thigh glucose uptake at both exercise intensities (r^2 = 0.23–0.35, p ≤ 0.003) and between GLUT4 abundance and thigh glucose extraction at 25% iPPO (r^2 = 0.14, p = 0.02; Suppl. Figs. 1–4 in Suppl. appendix). An association (r = 0.61 to 0.95) between training-induced increases in thigh glucose uptake and muscle GLUT4 abundance has also been reported in humans [6] and rats [49]. It should be noted, however, that not only GLUT4 abundance determines the capacity for transmembrane glucose disposal. Also its translocation and intrinsic activity at the cell surface, both of which can be facilitated independently by contraction and insulin, appear to be influential [50] and could have been augmented by BFR-training.

Intravenous infusion of antioxidant (NAC) attenuated thigh net glucose uptake during exercise only in the BFR-trained leg, which was primarily caused by a decrease in glucose extraction (Fig. 8–10). The attenuating effect of NAC was most pronounced during the first 3 min of exercise (Fig. 10B and D), where it accounted for approximately 50–100% of thigh net glucose uptake irrespective of intensity (Fig. 5B+D and Fig. 10B+D). These observations suggest that an increase in ROS-mediated glucose extraction contributes to improvements in glucose uptake by the musculature after training with BFR. Further, they indicate that the involvement of ROS was either an effect of augmented GLUT4 translocation, intrinsic activity, or both [48]. In line with the present findings, NAC depressed contraction-stimulated glucose uptake (as assessed by the tracer, 2-deoxy-D-glucose) by ~50% in mouse skeletal muscle ex vivo [24,32]. However, intravenous infusion of a similar dose of NAC as in the present study, which raised muscle glutathione and cysteine concentrations [34], had no effect on tracer-determined (6.6-^{2}H-glucose) glucose disposal during moderate-intensity (~62% VO_{2max}) exercise in recreationally-active men (VO_{2max} = 52 mL/kg/min) [38]. Accordingly, in the present study, NAC did not affect glucose uptake in the control leg at any time point or in the BFR-leg before training (Fig. 10). Thus, our results suggest that the contribution from ROS to muscle glucose regulation in humans may depend on the trained state of skeletal muscle.

BFR-training increased muscle reduced glutathione (GSH) concentration, and elicited a higher GSH to oxidised glutathione (GSSG) ratio compared to training without BFR, as reported in our companion article [39]. Further, only training with BFR elicited an increase in the abundance of Cu/Zn-SOD (~39%) and GPX-1 (~29%), whereas the abundance of Mn-SOD and catalase was not significantly altered by training in either leg (Fig. 11). Together with the observation of an attenuating effect

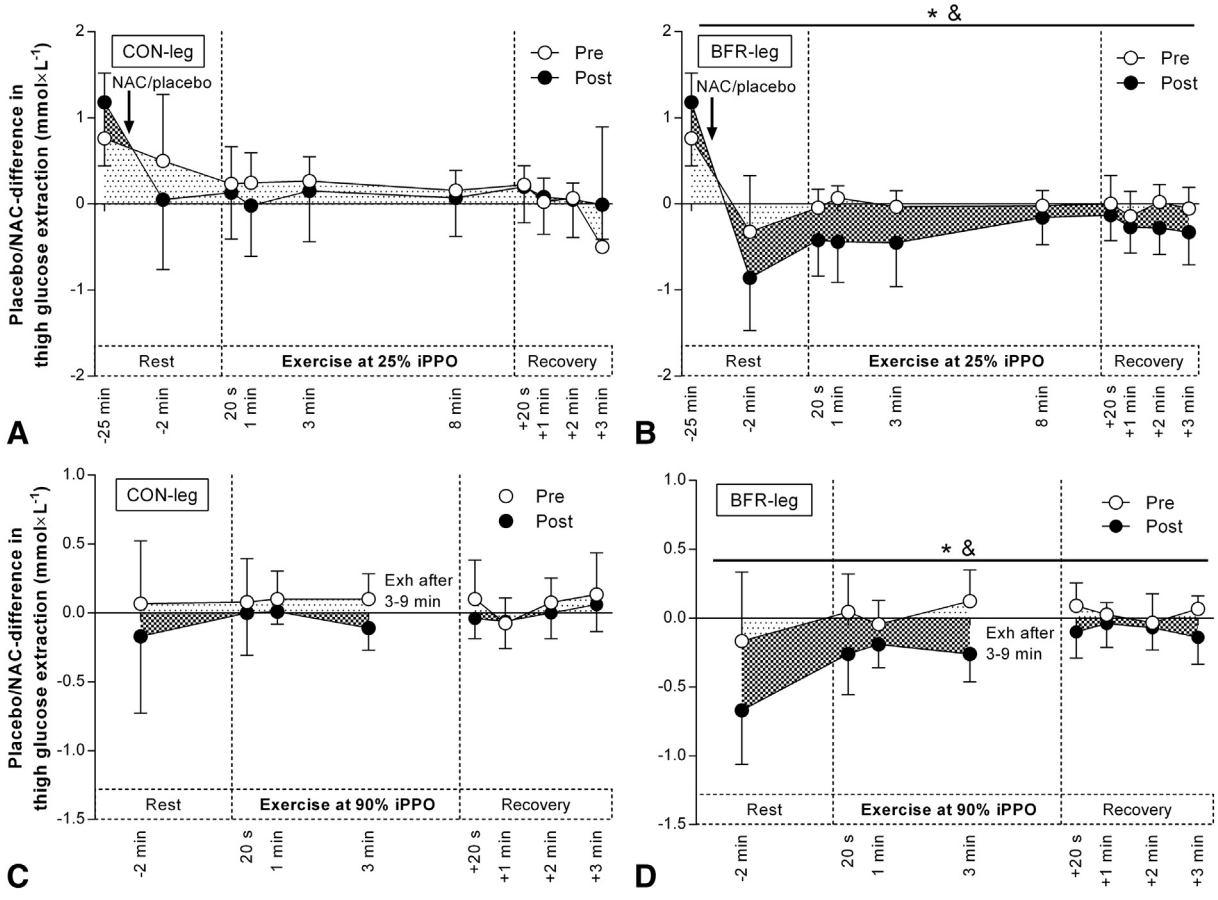

Fig. 9. Effect of *N*-acetylcysteine (NAC) infusion on thigh glucose extraction before and after training. A) Difference in thigh glucose extraction between NAC and placebo for the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Difference in thigh glucose extraction in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Difference in thigh glucose extraction between NAC and placebo for CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Difference in thigh glucose extraction between NAC and placebo for BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals. *p < 0.05, NAC lower than placebo at Post; &p < 0.05, NAC effect different from CON-leg at Post.

of NAC on net glucose uptake in the BFR-trained leg, these results indicate that increases in the capacity of specific antioxidant systems contributed to the higher glucose uptake in this leg after training, although correlations between the content of antioxidant enzymes and redox markers (GSH, GSSG, GSH/GSSG ratio, and total GSH content) in skeletal muscle on one hand, and thigh glucose uptake and extraction during exercise and the subsequent recovery on the other, were not significant (Suppl. Figs. 1–8, Suppl. Appendix). Both Cu/Zn-SOD and GPX-1 are located in the cytosol, where they scavenge superoxide and mainly hydrogen peroxide, respectively. In contrast, a key role of Mn-SOD and catalase is to scavenge mitochondrial-derived ROS [51,52]. Thus, the selective upregulation of Cu/Zn-SOD and GPX-1 in the BFR-trained leg could indicate that increasing the levels of non-mitochondrial ROS was particularly important for the attenuating effect of NAC and thus the enhancement of muscle glucose uptake at the onset of exercise after training with BFR. However, Mn-SOD abundance was higher in BFR-leg than in CON-leg after training, indicating BFR-training might also affect the mitochondrial capacity for scavenging of superoxide. Moreover, while BFR-training improved thigh net glucose uptake during exercise, NAC markedly attenuated this effect (Fig. 10), despite increasing further muscle GSH concentration and GSH:GSSG ratio [39]; thus pointing to a dose-dependent effect of antioxidant on muscle glucose uptake. On this basis, we speculate that manipulation with the function of cytosolic antioxidant systems, and thus the levels of ROS, works as a fine-tuning mechanism in regulating glucose uptake by exercising muscles in physically-trained individuals. In this regard, it should be noted that while participants' diet were controlled prior to the invasive days with antioxidant infusion, we cannot exclude that voluntary changes in diet throughout the study period affected antioxidant intake, despite we instructed the participants to keep their routine dietary pattern.

Another key observation was that training with BFR, in contrast to training without BFR, elevated the muscle's abundance of nNOS (~18%), indicating a greater potential for NO production. Furthermore, the higher abundance of Cu/Zn-SOD and GPX-1, evident only in the BFR-trained leg, may have reduced the amount of NO binding with superoxide and hydrogen peroxide, respectively, and thereby contributed to increase NO availability [19,41], although this latter possibility remains hypothetical. With the use of different nNOS inhibitors (e.g., L-NAME and L-NMMA), several human experiments have demonstrated a role of NO in contraction-stimulated glucose uptake by skeletal muscle [27–29,53,54]. Thus, our results support that an increased level of NO could have contributed to enhance thigh glucose uptake at the onset of exercise in the BFR-trained leg. Measurements of ROS/RNS levels in vivo and of the function of ROS/NO-producing systems (e.g., NADPH oxidase and nNOSμ) would provide further valuable insights on this topic.

The p-AMPK Thr^{172}/AMPK and p-ACC Ser^{79}/ACC ratios and p-ACC Ser^{79} remained unchanged with training in both legs, although the large inter-subject variation in these variables should be noted (Fig. 7). Further, p-AMPK Thr^{172} increased only after training with BFR (~38%; effect size = 0.85; Fig. 7). However, there was a comparable

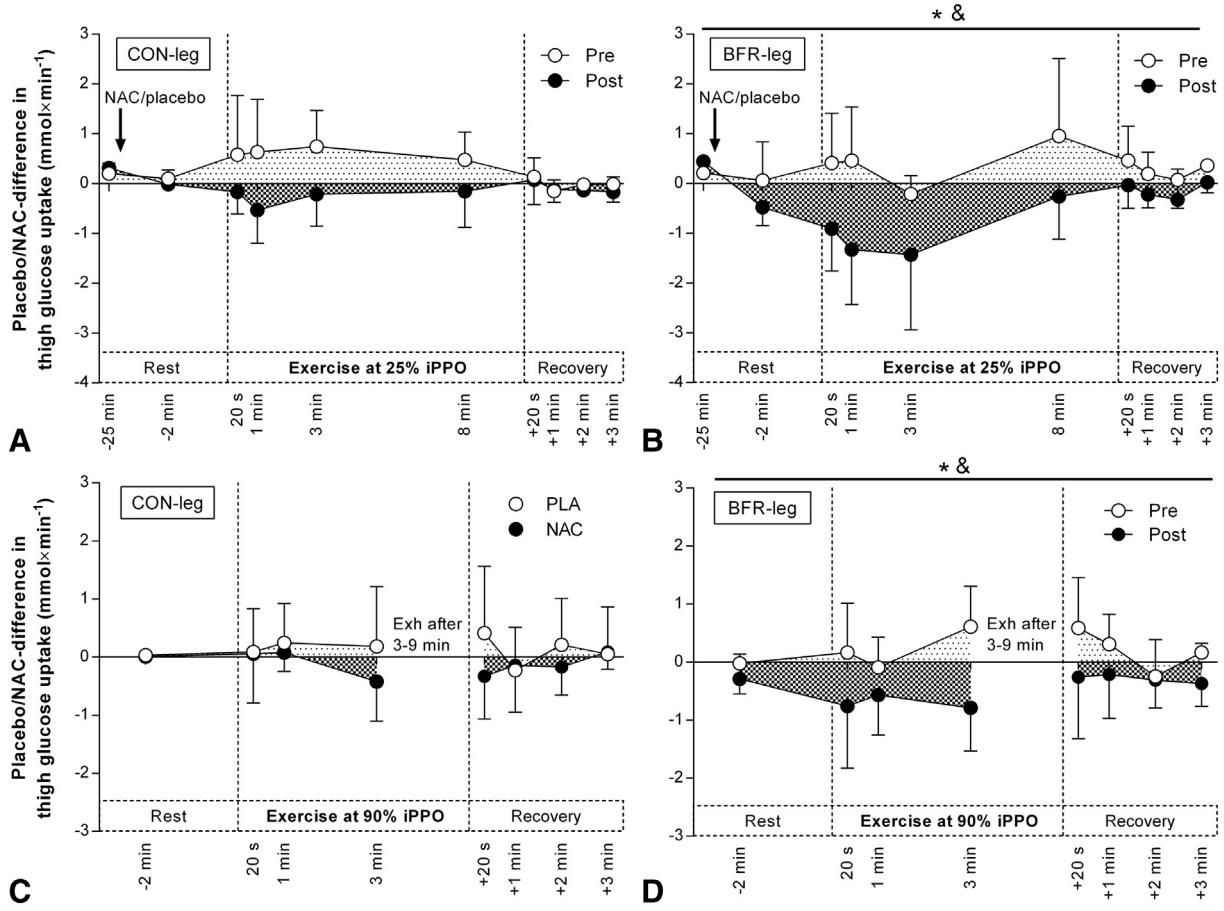

Fig. 10. Effect of *N*-acetylcysteine (NAC) infusion on thigh glucose uptake before and after training. A) Difference in thigh glucose uptake between NAC and placebo for the leg training without blood flow restriction (CON-leg, circles) during exercise at 25% peak aerobic power output (iPPO) before (Pre, white symbols) and after (Post, black symbols) training. B) Difference in thigh glucose uptake in the leg training with blood flow restriction (BFR-leg, squares) during exercise at the same intensity (25% iPPO). C) Difference in thigh glucose uptake between NAC and placebo for CON-leg during exercise at 90% iPPO to exhaustion (Exh). D) Difference in thigh glucose uptake between NAC and placebo for BFR-leg during exercise at the same intensity (90% iPPO). n = 10. The arrow indicates the start of the infusion. Data are expressed as means ± 95% confidence intervals. *p < 0.05, NAC higher than placebo in BFR-leg at Pre; &p < 0.05, NAC lower than placebo in BFR-leg at Post.

mean increase (~51%) and effect size (0.70) in CON-leg. Together, these results question the involvement of AMPK Thr^{172} modulation in causing the higher net rate of muscle glucose uptake after training with BFR. Accordingly, in mice, NO-induced stimulation of muscle glucose uptake was independent of AMPK and its phosphorylation at Thr^{172} [24,26]. Treatment of human vastus lateralis muscle samples ex vivo with the NO donor, spermine NONOate, elicited concomitant increases in glucose uptake and AMPK α_1 activity (1.7 fold) [55], raising the possibility that increased activity of specific AMPK complexes, which may have been left undetected by measurement of muscle global AMPK phosphorylation in the present study [56], could be important for increases in muscle glucose uptake in humans. This is supported by findings in a number of animal studies [32,57], although other studies do not support this hypothesis [58]. In addition, we found that the attenuating effect of NAC on the net rate of glucose uptake in the BFR-trained leg was also unrelated to changes in resting muscle p-AMPK Thr^{172}/AMPK and p-ACC Ser^{79}/ACC ratios (Fig. 7). Consistent with these observations, NAC-induced depression [24], as well as ROS-induced stimulation [58], of glucose uptake in animal skeletal muscle was independent of AMPK phosphorylation at Thr^{172}, and AMPK α_1 and α_2 activity, respectively, although this is not a universal finding [32].

5.3. Strengths and weaknesses

The strengths of the present study are: (1) The within-subject, unilateral training model that allowed two treatments (BFR vs. control) matched for training volume to be studied concurrently within the same participant, thereby increasing the statistical power vs. a randomised, two-group design. Our design also ensured examination of the muscular mechanisms in isolation from systemic factors. (2) The experiments undertaken before and after the training period allowed glucose regulation to be studied (with and without intravenous antioxidant infusion) directly over the exercising muscles that were primarily engaged during training sessions. This was made possible by insertion of catheters into the femoral artery and vein of both legs/treatment arms, and the use of ultrasound Doppler and single-leg, knee-extensor exercise model. On the other hand, there are a number of limitations that should be highlighted. The

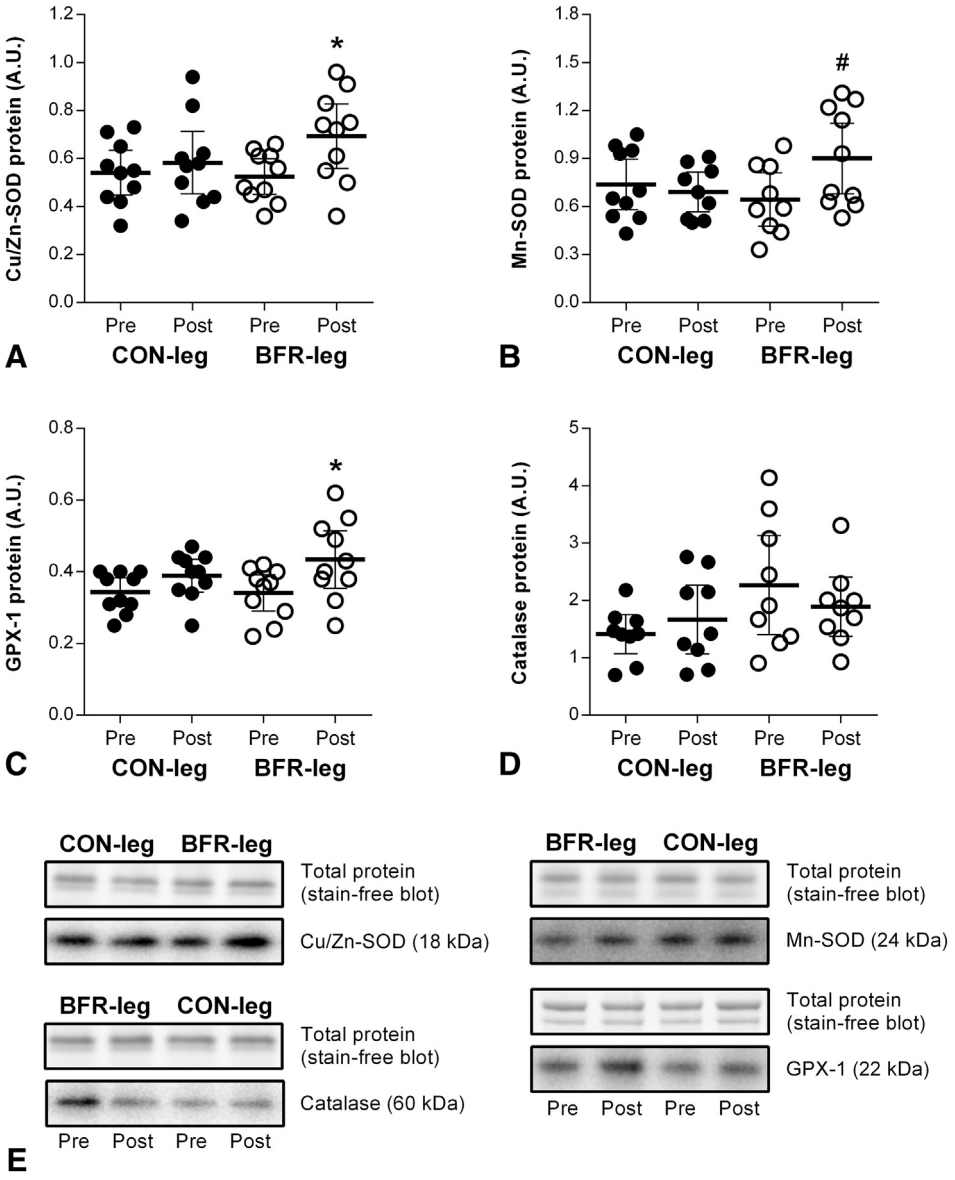

Fig. 11. Effect of training with and without blood flow restriction (BFR) on antioxidant enzyme abundance in human muscle. A) Cu/Zn-SOD, B) Mn-SOD, C) GPX-1, and D) Catalase, abundance before (Pre) and after (Post) six weeks of training without (CON-leg) and with blood flow restriction (BFR-leg). E) Representative western blots of the enzymes and total protein loaded onto the stain-free gels. Data are expressed as means ± 95% confidence intervals and individual values are shown either as solid circles for CON-leg or open circles for BFR-leg. *$p < 0.05$, higher Post than Pre in BFR-leg. #$p < 0.05$, higher in BFR-leg than in CON-leg at Post.

large inter-subject variation in muscle initial concentrations of glutathione [39] may have caused varied responses among participants to NAC infusions, because NAC is a precursor of glutathione. This likely contributed to the large variation in effect of NAC on thigh glucose uptake among participants. Further, the fixed order of infusions on experimental days (first placebo, then NAC) could ultimately have caused an order effect. However, the order was not randomised, because the reduced form of NAC in blood has a half-life of almost 7 h [43]. Further, infusions were performed on the same day, because a doubling of such invasive, experimental days would be unsuitable to complete for participants (i.e., unethical). In addition, large inter-subject variation in protein changes to training should also be noted.

5.4. Conclusions

In contrast to work-matched training without BFR, training with BFR markedly enhanced thigh net glucose uptake during exercise in men, which was caused by an increase in thigh glucose extraction. Further, exercise performance (time to exhaustion) increased only in the BFR-trained leg. Several mechanisms, such as an altered antioxidant capacity, augmented ROS-mediated glucose extraction, as well as an increased GLUT4 abundance and NO availability, may have contributed to the higher glucose uptake during exercise. On the other hand, the enhancement of muscle glucose uptake by BFR-training occurred independent of changes in resting muscle p-AMPK Thr^{172}/AMPK and p-ACC Ser^{79}/ACC ratios. Because glucose regulation is compromised in people with life-style-related diseases, such as type-2 diabetes [12,59], and may become a limiting factor for the performance of endurance athletes [60], future research should examine the potential benefits of BFR-training on glucose metabolism in these populations.

5.5. Translational perspective

Because carbohydrate metabolism is limiting for performance in many sports, there is a great practical interest in strategies that can increase the reliance on carbohydrate metabolism for energy production during exercise in athletes. In active men, a prior decline in muscle glycogen content from ~96 to 54 mmol/kg wet weight muscle reduces the contribution from carbohydrate metabolism to ATP resynthesis during exercise (40 min at 70% VO_{2max}) [61]. However, with the increase in glucose uptake by exercising muscles after BFR-training in the present study (Fig. 5), there would seem an increased potential to utilize glucose for ATP resynthesis, even when muscle glycogen is substantially reduced (or depleted). This could be advantageous in sports, where muscle glycogen becomes a limiting factor for performance, such as towards the end of a soccer match, a marathon, or a stage in the Tour de France. Future studies should thus examine the effects of BFR-training on glycogen utilization and performance in athletes engaged in these sports.

Despite the reported benefits of high-intensity interval training for type-2 and pre-diabetic patients (e.g., lowering postprandial hyperglycaemia) [14–16], there is a general concern about the safety of this training regimen for these patients due to an increased risk of cardiovascular events [17]. The present study presents an effective alternative to improve glucose metabolism in man in the form of interval cycling with BFR, where training load is moderate (60–80% of W_{max}) and the duration is low (only 54 min of exercise per week).

Declaration of Competing Interest

The authors have no conflict of interests that relate to the content of this manuscript.

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Author contributions

DC and JB conceived the study. DC, MH, and JB designed experiments. DC, KE, and MH collected data. DC and KE analysed blood flow data. DC performed the other data and muscle analyses and drafted the manuscript. All authors contributed to data interpretation, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the manuscript in ensuring that questions related to the accuracy or integrity of any part of the manuscript are appropriately investigated and resolved.

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Supplementary data

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