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Dietary nitrate accelerates postexercise muscle metabolic recovery and O_2 delivery in hypoxia

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Abstract

We tested the hypothesis that the time constants (τ) of postexercise T2* MRI signal intensity (an index of O_2 delivery) and muscle [PCr] (an index of metabolic perturbation, measured by ^{31}P-MRS) in hypoxia would be accelerated after dietary nitrate (NO_3^-) supplementation. In a double-blind crossover design, eight moderately trained subjects underwent 5 days of NO_3^- (beetroot juice, BR; 8.2 mmol/day NO_3^-) and placebo (PL; 0.003 mmol/day NO_3^-) supplementation in four conditions: normoxic PL (N-PL), hypoxic PL (H-PL; 13% O_2), normoxic NO_3^- (N-BR), and hypoxic NO_3^- (H-BR). The single-leg knee-extension protocol consisted of 10 min of steady-state exercise and 24 s of high-intensity exercise. The [PCr] recovery τ was greater in H-PL (30 ± 4 s) than H-BR (22 ± 4 s), N-PL (24 ± 4 s) and N-BR (22 ± 4 s) ($P < 0.05$) and the maximal rate of mitochondrial ATP resynthesis (Q_{max}) was lower in the H-PL (1.12 ± 0.16 mM/s) compared with H-BR (1.35 ± 0.26 mM/s), N-PL (1.47 ± 0.28 mM/s), and N-BR (1.40 ± 0.21 mM/s) ($P < 0.05$). The τ of postexercise T2* signal intensity was greater in H-PL (47 ± 14 s) than H-BR (32 ± 10 s), N-PL (38 ± 9 s), and N-BR (27 ± 6 s) ($P < 0.05$). The postexercise [PCr] and T2* recovery τ were correlated in hypoxia ($r = 0.60$; $P < 0.05$), but not in normoxia ($r = 0.28$; $P > 0.05$). These findings suggest that the NO_3^--NO_2^--NO pathway is a significant modulator of muscle energetics and O_2 delivery during hypoxic exercise and subsequent recovery.

Keywords: hypoxia, skeletal muscle, phosphocreatine, nitric oxide, nitrite

SKELETAL MUSCLE HYPOXIA is a hallmark of disease conditions where O_2 delivery to the periphery is impaired (44). Detrimental effects of hypoxia on muscle function include a reduction in the maximal rate of muscle oxidative ATP resynthesis (Q_{max}) (26), which is reflected as a reduction in maximal pulmonary O_2 uptake ($\dot{V}O_{2max}$; 54), and an accelerated rate at which the limited "anaerobic" energy substrates are utilized during muscular contraction (23, 25, 65). We recently reported that dietary supplementation with inorganic nitrate (NO_3^-) ameliorated the detrimental effects of acute normobaric hypoxia on muscle metabolic responses to exercise and exercise tolerance in humans (65). These effects were attributed to an elevated potential for nitric oxide (NO) production via stepwise reduction of NO_3^- to bioactive nitrite (NO_2^-) (45).

Low NO bioavailability is associated with poor vascular function and limited exercise capacity (39, 53). NO modulates mitochondrial O_2 consumption (59) and acts as the primary metabolic stimulus for vascular endothelial relaxation during hypoxic exercise (8, 9). The reduction of NO_2^- to NO in the stomach, tissues, and the systemic circulation is increased under conditions of reduced O_2 availability (45, 49). NO may also enhance the local matching of O_2 availability to metabolic rate (19, 60, 63). We showed previously that

nitrate supplementation, which significantly elevated the circulating [NO_2^-], accelerated the rate of muscle phosphocreatine (PCr) recovery in hypoxia, suggesting that the maximal rate of mitochondrial ATP resynthesis following exercise was restored to a similar level in moderate hypoxia (F_{IO2} = 0.145) as was possible in normoxia (65). Nitrate supplementation also attenuated the rate of PCr degradation and inorganic phosphate (P_i) accumulation during exhaustive, severe-intensity exercise in hypoxia (65). Elevated circulating [NO_2^-] following nitrate supplementation appeared to increase the skeletal muscle resilience to the bioenergetic challenge of hypoxic exercise (65), an effect that may be related, in part, to the vasodilatory effects of NO and/or nitrite (46).

One possible mechanism underlying the faster PCr recovery in hypoxia after nitrate supplementation is increased mitochondrial efficiency (37). However, PCr recovery kinetics is not accelerated by nitrate supplementation in normoxia (17), suggesting that increased blood flow and/or better matching of local perfusion to metabolic rate may be more important determinants of faster PCr recovery in hypoxia than increased mitochondrial efficiency. Considering the powerful vasodilatory influence of NO, the elevated potential for NO production following nitrate supplementation might improve O_2 delivery to muscle during recovery from exercise in hypoxia. In our previous study (65), however, we did not compare nitrate and placebo conditions in normoxia or measure indexes of O_2 delivery.

In the present study, therefore, we examined changes in the effective transverse relaxation time (T2*) weighted magnetic resonance (MR) signal during exercise and subsequent recovery. The T2* MR signal intensity is a function of the blood flow into the volume of muscle under investigation combined with the magnetic properties of blood and tissue, in particular those deriving from the oxygenation status. Therefore, T2* signal increase can be a consequence of an increase in blood volume and/or an increase in the proportion of oxygenated blood due to the diamagnetic properties of oxyhemoglobin. Previous studies have indicated that the increase in T2* MR signal intensity reflects the increase in inflow of freshly oxygenated blood into the muscle during exercise, as well as during postexercise recovery and postischemic reactive hyperemia (42, 58). Several studies also indicate a close relationship between changes in MR signal intensity and quantitative perfusion measurements (40, 41, 61, 62). It is important to note, however, that the T2* weighted MRI does not allow direct quantification of perfusion and is influenced by a range of additional tissue parameters to those discussed above (43). However, the significant advantage of this noninvasive technique is high temporal and spatial resolution of data acquisition, which is essential for the kinetic analysis of the postexercise response.

The purpose of this study was to address the mechanistic bases for the effects of dietary nitrate on muscle energetics during hypoxic exercise and recovery. It was hypothesized that nitrate supplementation will *1*) augment the rate of change of the postexercise T2* MR signal compared with placebo; and *2*) accelerate the [PCr] recovery kinetics compared with placebo in hypoxia but not in normoxia.

METHODS

Ethical approval. The study was approved by the University of Exeter research ethics committee and was conducted in accordance with the standards set by the Declaration of Helsinki. Subjects gave written informed consent to participate after the experimental procedures, associated risks, and potential benefits of participation had been explained.

Subjects. Eight healthy, moderately trained subjects volunteered to participate in this study (5 males, mean ± SD: age 27 ± 10 years, body mass 82.4 ± 7.5 kg, height 1.77 ± 0.04 m; 3 females: age 27 ± 7 years, body mass 61.5 ± 8.7 kg, height 1.70 ± 0.08 m). Subjects were asked to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each test. Participants were asked to refrain from consuming caffeine for 6 h and alcohol for 24 h before each test. Subjects also abstained from using antibacterial mouthwash throughout the study to preserve commensal oral bacteria, which reduce NO_3^- to NO_2^- (30). Subjects were instructed to avoid foods rich in nitrate (such as leafy green vegetables and beetroot) during the study period.

Experimental procedures. Exercise tests were performed in a prone position within the bore of a 1.5 T superconducting magnet (Gyroscan Clinical Intera, Philips) using a custom-built non-ferrous ergometer. The right foot was fastened securely to a padded foot brace and connected to the ergometer load baskets by a

rope and pulley system. One-legged knee extensions of the right leg (range of motion of ~0.22 m) were performed such that two knee extensions were carried out within 3 s followed by 1 s of rest (work:rest ratio of 3:1).

Subjects were initially familiarized with the test protocol on a separate day prior to data collection. During this visit, an intermediate work rate, which resulted in steady-state muscle PCr response and could be sustained for 10 min without duress, was determined for each subject. Subsequently, a work rate for a 24-s high-intensity exercise bout (which was used for the assessment of PCr recovery kinetics) was determined as the highest work rate that the subject could maintain for 24 s without compromising the timing and range of motion of the knee-extension task. The high work rate and short duration for this 24-s protocol have been optimized for the modeling of [PCr] recovery kinetics (26), with the objective of providing a marked reduction in [PCr] in a short period of time while the pH does not fall below the resting baseline.

The work rates determined for the 10-min and 24-s bouts for each subject during the familiarization visit were used during all subsequent experimental visits. The exercise protocol during T2* scanning visits consisted of a 10-min bout of steady-state exercise and, following 10 min of passive rest, one 24-s bout of high-intensity exercise. The exercise protocol during the ^{31}P-MRS visits included a 10-min bout of steady-state exercise, 10 min of passive rest, and two 24-s bouts of high-intensity exercise which were separated by 3 min 36 s of passive rest. Knee extensor displacement was measured using a calibrated optical shaft encoder (Type BDK.06.05A 100-5-4; Baumer Electric, Swindon, UK) connected to the weight basket pulley, and load was measured using an aluminum load cell (Type F250EBR0HN, Novatech Measurements, St. Leonards-on-Sea, East Sussex, UK). Work done was calculated as the product of force and displacement.

Subjects wore a facemask and breathed the normoxic or hypoxic inspirate for a minimum of 30 min prior to the start of the exercise protocol while resting within the bore of the scanner. The subjects then continued to breathe the given inspirate throughout the exercise protocol and subsequent recovery. The inspirate was generated using a CAT filtration system (Sporting Edge UK, Basingstoke, UK). The generator fed via an extension tube to a 150-liter Douglas Bag (Cranlea, Birmingham, UK) placed within the scanner room. This acted as a reservoir and mixing chamber and had a separate output pipe feeding into a two-way breathing valve system (Hans Rudolf, Cranlea), which was connected to the facemask. The O_2 and CO_2 concentration of the inspirate was monitored during each test using a Servomex 5200 High Accuracy Paramagnetic O_2 and CO_2 Analyzer (Servomex, Crowborough, UK). The gas analyzer was calibrated prior to each test with a 16.0% O_2, 8.0% CO_2, and 76.0% N gas mix (BOC Special Gases, Guildford, UK). The F_{IO2} measured during the hypoxic tests was 0.132 ± 0.001.

Blood pressure of the brachial artery was measured with the subject remaining in the prone position, immediately prior to the start of the exercise protocol (Schiller Maglife Light, Siemens, Germany). Four measurements were taken and the mean value of the last three was recorded. Heart rate and arterial O_2 saturation (Sa_{O_2}) were monitored continuously throughout each testing session with a finger probe oximeter (Nonin 7500FO, Nonin Medical, Plymouth, MN).

Supplementation and nitrite analyses. Subjects underwent dietary supplementation with NO_3^--rich beetroot juice (BR) and NO_3^--depleted beetroot juice (PL) in a double-blind, counterbalanced, randomized order separated by a minimum of 5-day washout period. The experimental conditions were *1*) normoxic placebo (N-PL), *2*) hypoxic placebo (H-PL), *3*) normoxic beetroot (N-BR), and *4*) hypoxic beetroot (H-BR).

For each experimental condition, supplementation began 3 days prior to the first exercise test with subjects consuming 0.5 l/day of BR containing 8.2 mmol of nitrate or 0.5 l/day of PL containing 0.003 mmol nitrate (Beet It, James White Drinks, Ipswich, UK). The supplement was taken in two equal doses of 0.25 liter in the morning and evening on *days 1–3*, whereas on testing days, 0.5 liter was ingested 2.5 h prior to the start of the exercise test. On *day 4*, the subject performed the exercise protocol for the assessment of T2* data, and on *day 5* the same exercise protocol was repeated for the acquisition of ^{31}P-MRS data.

Upon arrival at the laboratory during each visit, a venous blood sample (5 ml) was drawn from the antecubital vein into a lithium-heparin tube (Vacutainer, Becton Dickinson). Samples were centrifuged at 2,700 *g* and 4°C for 10 min within 3 min of collection. Plasma was extracted and immediately frozen at −80°C, for later analysis of [NO_2^-] using a modification of the chemiluminescence technique as previously

described (65).

MRI and MRS protocols. On days when T2 measurements were undertaken, subjects had a four-element body coil wrapped around their upper legs, with the two anterior elements secured within the scanner bed and the two posterior elements placed on the back of the legs and fixed with Velcro straps, which also served to restrict subject motion. The scanning procedure consisted initially of low-resolution survey images. A series of 20 high-resolution (resolution 1 × 1 mm in plane, 5 mm slice thickness) slices using a T1 (longitudinal relaxation time) weighted turbo spin echo (TSE) sequence were then obtained in the coronal plane to allow identification of the lateral and medial condyles. On the first visit, a single transverse slice location, approximately midthigh, was selected, and its position relative to the condyles was measured. This slice position was replicated for measurements in all future visits.

Prior to the exercise protocol, both resting muscle T2 (transverse relaxation time) and T2* (effective transverse relaxation time) were determined. T2 was calculated by running a spin echo sequence with 8 echoes. An in-plane resolution of 0.9 × 0.9 mm, with a 5-mm slice thickness was used, with a minimum TE (echo time) of 13 ms and each subsequent echo being obtained at a time equal to 13 × n ms. T2* was calculated using a gradient echo sequence, with all timing and resolution parameters equal to those for the T2 measurement.

During the exercise protocol and subsequent recovery T2* acquisition was continuously undertaken using a single shot echo planar (EPI) sequence with a TE of 35 ms, a slice thickness of 5 mm, an in-plane resolution of 1.3 × 1.4 mm and sampling repeated (repetition time TR) every 4 s. The sequence was timed such that image acquisition was obtained during the rest period of the work-rest cycle. A spectrally and slice selective binomial excitation fat suppression pulse was used to decrease the signal from subcutaneous fat. After 10 dummy scans had been run to ensure that tissue signal had reached equilibrium, 396 dynamic scans were acquired.

On days when ^{31}P-MRS measures were made, absolute concentrations of muscle metabolites were established prior to the exercise protocol using a calibrated ^{31}P-MRS technique and resting unsaturated spectra as described previously (65). For the exercise protocol, subjects were positioned in the same location as for the T2* data acquisition and ^{31}P spectra were acquired continuously during exercise and subsequent recovery using a 6 cm ^{31}P surface coil. The general scanning parameters were selected as described previously (60) with data acquired every 1.0 s with four phase cycles leading to a spectrum being acquired every 4 s.

MRI data analysis. To determine T2 and T2* and assess the temporal changes in T2* signal in specific muscle locations, separate regions of interest (ROI) were manually drawn on the acquired images within the rectus femoris muscle and vastus medialis muscle of the right leg (active) and the rectus femoris muscle of the left leg (inactive). The rectus *femoris* muscle is the prime mover for this exercise mode which occurs over a limited range of motion (22 cm).

T2 at rest was calculated based upon the relationship:

$$S = S_0 \exp\left(-t/\mathrm{T2}\right),$$

where S is the measured signal, S_0 is the equilibrium signal and t is the echo time. Signal intensity was recorded for each ROI for all eight echoes and a plot of ln(S) against t resulted in a least square fit gradient equal to −1/T2. Likewise, T2* was calculated based upon the relationship:

$$S = S_0 \exp\left(-t/\mathrm{T2}^*\right)$$

In a similar manner to the T2 calculation, T2* at rest was calculated via the determination of −1/T2* obtained from the gradient of the plot of ln(S) against t.

The baseline T2* signal intensity was calculated as the mean T2* signal over the 2 min immediately preceding the commencement of exercise for both the active and inactive leg. The amplitude of the T2* signal on-kinetics was calculated as the difference between the baseline and the mean T2* signal over the last 24 s of the 10-min exercise bout. The end-exercise T2* signal of the inactive leg was calculated in the same manner and the amplitude was calculated as the difference between the end-exercise and baseline values. The transient T2* increase during subsequent recovery from exercise in the active leg (i.e., off-kinetics) was also characterized by fitting a single-exponential function to T2* signal data recorded over 90 s following the 10-min exercise bout and the 24 s exercise bout (see Fig. 5). Each recovery was fitted separately and the mean of the two τ's for T2* signal off-kinetics from the 10-min and 24-s bouts was calculated for each subject.

^{31}P-MRS data analysis. The spectra were quantified via peak fitting using the jMRUI (version 3) software package employing the AMARES fitting algorithm (64). Intracellular pH was calculated using the chemical shift of the P_i spectral peak relative to the PCr peak. [ADP] was calculated via knowledge of [P_i], [PCr], and pH values, taking into account the dependency of rate constants on pH (33).

Resting values of PCr, ATP, P_i and pH at resting baseline were determined from the unsaturated spectra acquired prior to the start of the exercise protocol and the end-exercise values were taken as the mean values measured over the final 24 s of exercise. Phosphorylation potential was calculated as [ATP]/[ADP]·[P_i]. The Gibbs free energy of ATP hydrolysis (ΔG) was calculated as previously described taking into account the dependency of ΔG on pH (33). The maximal oxidative ATP turnover rate (Q_{max}) was calculated as (1/PCr recovery τ)·([PCr] baseline).

The [PCr] τ for the on-transient during the 10-min exercise bout was established by exponential modelling. With the exception of five tests (2 in H-PL and 3 in H-BR), the data conformed to a monoexponential function and were fitted through the entire 600 s. In the five data sets which showed a PCr "slow component," the fitting window was constrained to an initial start point of 60 s and extended incrementally thereafter until there was a clear departure of the measured data from the model fit, as judged from visual inspection of a plot of the residuals. The magnitude of a slow component was calculated as the difference between the asymptotic amplitude of the fundamental response and the mean value measured over the last 24 s of exercise for that condition. This approach enabled the best-fit exponential for the fundamental component of the response to be established irrespective of the presence of the slow component. The PCr recovery (off-kinetics) τ was determined by fitting a single-exponential function to the [PCr] recorded over 150 s following the two 24-s exercise bouts (Graphpad Prism, Graphpad Software, San Diego, CA). Each transition was fitted separately and the mean of the two τ's was calculated for each subject.

Statistical analyses. Two-way repeated-measures analyses of variance (supplement by F_{IO2}) were used to assess differences across the treatments (N-PL, H-PL, N-BR, and H-BR trials), and significant main or interaction effects were followed-up by LSD pairwise comparisons (v15.0, SPSS, Chicago, IL). Relationships between variables were assessed using Pearson's product-moment correlation coefficients. Statistical significance was accepted at the level of $P < 0.05$, and statistical trend was defined as $P < 0.10$. Data are presented as means ± SD unless stated otherwise.

RESULTS

The work rates for the exercise tests were 21 ± 1 W for the 10-min steady-state exercise bout and 30 ± 2 W for the 24-s high-intensity bouts. During the exercise bouts the mean Sa_{O_2} was lower in H-PL (85 ± 2%) and H-BR (86 ± 3%) compared with N-PL (97 ± 1%) and N-BR (97 ± 1%; $P < 0.05$).

Plasma [NO_2^-]. Plasma [NO_2^-] was greater in N-BR than N-PL prior to the T2* test ($P < 0.05$) and tended to be greater prior to the ^{31}P-MRS test ($P = 0.08$) (Fig. 1). Plasma [NO_2^-] was greater in H-BR than H-PL prior to the T2* and ^{31}P-MRS tests ($P < 0.05$) (Fig. 1).

The [PCr] and T2* signal off-kinetics. The end-exercise muscle [PCr] during the 24-s high-intensity exercise bout was not different between conditions (N-PL 24.0 ± 4.4 mM; H-PL 24.4 ± 4.3 mM; N-BR 24.5 ± 3.6 mM; H-BR 22.1 ± 3.3 mM; $P > 0.05$). The end-exercise pH in the 24-s exercise bouts (N-PL 7.11 ± 0.04; H-PL 7.11 ± 0.04; N-BR 7.09 ± 0.02; H-BR 7.13 ± 0.04) was not different between conditions or from resting baseline within each condition ($P > 0.05$ for all). The [PCr] recovery τ was greater in H-PL (30 ± 4 s)

compared with H-BR (22 ± 4 s), N-PL (24 ± 4 s), and N-BR (22 ± 4 s; $P < 0.05$ for all; Fig. 2*A*). The Q_{max} was lower in the H-PL (1.12 ± 0.16 mM/s) compared with H-BR (1.35 ± 0.26 mM/s), N-PL (1.47 ± 0.28 mM/s), and N-BR (1.40 ± 0.21 mM/s) conditions ($P < 0.05$; Fig. 2*B*). The PCr τ could not be determined in one subject in the H-BR condition due to low signal:noise resulting from excessive movement during the ^{31}P-MRS data acquisition. Therefore, the PCr recovery τ and Q_{max} data for H-BR are for $n = 7$.

The τ of T2* signal off-kinetics in H-PL (47 ± 14 s) was significantly greater compared with H-BR (32 ± 10 s), N-PL (38 ± 9 s), and N-BR (27 ± 6 s; $P > 0.05$ for all; Fig. 2*C*). The τ of T2* signal off-kinetics was also greater in N-PL compared with N-BR ($P < 0.05$). The [PCr] recovery τ was correlated with the τ of T2* signal off-kinetics in hypoxia ($r = 0.60$; $P < 0.05$; $n = 15$), but not in normoxia ($r = 0.28$; $P > 0.05$; $n = 16$).

Muscle metabolism at rest and during steady-state exercise. The resting [PCr], [P_i], [ADP], and ΔG were significantly lower and the phosphorylation potential was higher at rest following nitrate supplementation compared with placebo in both normoxia and hypoxia ($P < 0.05$ for all; Fig. 3). Muscle pH at rest was not different between conditions ($P > 0.05$). The resting [PCr] was negatively correlated with the plasma [NO_2^-] across all four conditions ($r = -0.35$, $P < 0.05$; $n = 32$). No relationships were detected between [PCr] recovery τ and the baseline [PCr] ($r = 0.30$, $P = 0.11$) or the baseline phosphorylation potential ($r = -0.26$; $P = 0.15$) ($n = 31$ for all).

Muscle metabolic responses to 10 min of steady-state exercise are illustrated in Fig. 4. The end-exercise [ADP] was lower in N-BR compared with N-PL and H-PL ($P < 0.05$), and the end-exercise ΔG was lower and the phosphorylation potential was higher in N-BR compared with N-PL ($P < 0.05$; Fig. 4; Table 1). The amplitude (i.e., difference from baseline to end-exercise) of the [PCr] response was smaller in N-BR and H-BR compared with N-PL ($P < 0.05$; Table 1).

T2* signal intensity during 10 min of steady-state exercise. The tissue water transverse relaxation time, T2, measured at resting baseline was smaller in H-PL (40.9 ± 2.2 ms) than in N-PL (42.6 ± 2.4 ms) and N-BR (42.7 ± 2.6 ms), and the T2 in H-BR (41.5 ± 2.0 ms) was also lower than in N-PL ($P < 0.05$ for all). There were no significant differences in the resting T2* (i.e., effective transverse relaxation time) between conditions (N-PL 25.8 ± 1.4 ms; N-BR 25.1 ± 1.4 ms; H-PL 25.2 ± 1.3 ms; H-BR 25.1 ± 0.8 ms).

The T2* signal intensity during the entire exercise protocol is shown in Fig. 5. The amplitude of the T2* signal during 10 min of steady-state exercise was greater in H-PL (32.5 ± 7.1%) compared with N-PL (28.3 ± 7.7%) and N-BR (25.1 ± 8.7%; $P < 0.05$; Fig. 5). The T2* signal amplitude also tended to be greater in H-PL than H-BR (28.5 ± 11.2%; $P = 0.09$) and the amplitude in H-BR was not different from the normoxic conditions ($P > 0.05$). The differences in T2* signal amplitude between H-PL and N-PL, and H-PL and H-BR were inversely correlated with the differences in Q_{max} between the same conditions ($r = -0.60$, $P < 0.05$, $n = 15$).

The T2* signal measured during the exercise protocol in the vastus medialis muscle of the active right leg decreased during the exercise protocol by 4.3% in N-PL, 3.8% in N-BR, 1.5% in H-PL, and 3.4% in H-BR ($P < 0.05$ compared with baseline in all conditions). Likewise, the T2* signal intensity from the rectus femoris muscle of the inactive left leg decreased from baseline to the end of exercise protocol by 2.1% in N-PL, 2.8% in N-BR, 1.3% in N-BR, and 1.6% in H-BR ($P < 0.05$ compared with baseline in all conditions).

There were no significant differences between conditions in resting systolic or diastolic blood pressure (Table 2). Heart rate at rest and at the end of exercise was higher in hypoxia compared with normoxia ($P < 0.05$; Table 2).

DISCUSSION

The primary novel findings of this study were that *1*) nitrate supplementation resulted in accelerated postexercise T2* signal intensity kinetics in both normoxia and hypoxia, and *2*) the faster [PCr] recovery kinetics in hypoxia following nitrate supplementation was related to the greater rate of change in postexercise T2* signal. In addition, the resting muscle PCr concentration and ΔG were reduced and the phosphorylation potential was increased after nitrate supplementation. Our data suggest that the faster [PCr] recovery kinetics after nitrate supplementation in hypoxia may be linked to enhanced O_2 delivery in the immediate postexercise period, while the increase in muscle phosphorylation potential implies that mitochondrial

efficiency may have been improved following nitrate supplementation. The significant beneficial influence on muscle O_2 delivery and energy metabolism by dietary nitrate during hypoxic exercise and subsequent recovery has clear translational implications for improving exercise tolerance (65) and functional capacity in conditions where muscle O_2 delivery is impaired.

PCr recovery kinetics. Nitrate supplementation did not alter PCr recovery kinetics in normoxia. However, in hypoxia (F_{IO2} ~0.13; Sa_{O_2} ~86%), nitrate supplementation accelerated PCr recovery, such that the τ was not different from that measured under normoxic conditions (SaO_2 ~97%). The PCr recovery is slowed in hypoxia due to the lower O_2 pressure gradient between the microcirculation and the myocyte (21, 23, 50). In normoxia, possible mechanisms by which the [PCr] recovery rate might be accelerated include increased mitochondrial mass and improved mitochondrial efficiency (12). However, although improved mitochondrial efficiency has been reported following nitrate supplementation in humans (37), we have not observed faster [PCr] recovery kinetics in normoxia following nitrate supplementation (17, 36, present study). As expected, the T2 (transverse relaxation time) measured at resting baseline was suppressed by hypoxia, indicative of reduced hemoglobin saturation. The T2* (effective transverse relaxation time), which was measured during exercise and subsequent recovery, was elevated throughout the protocol and represented slower postexercise recovery kinetics in the H-PL condition compared with normoxia. It should be noted that while the precise determinants of skeletal muscle T2* signal change during exercise and recovery are known to be multifactorial and to some extent paradigm dependent, current knowledge suggests that the T2* may be considered to reflect perfusion related changes in oxygenation of the vascular compartment (27). The faster postexercise T2* signal kinetics in H-BR compared with H-PL (Fig. 2*C*) may be indicative of enhanced O_2 driving pressure into the cell during the immediate recovery (i.e., restoring O_2 gradient to a similar level as in the normoxic conditions), thus enabling a faster rate of mitochondrial ATP resynthesis and, therefore, faster PCr resynthesis rate in H-BR compared with H-PL. This interpretation is supported by the positive correlation between the [PCr] τ and T2* τ which was observed in hypoxia ($r = 0.60$) but not in normoxia ($r = 0.28$). It is important to note that changes in muscle metabolic control (discussed below) consequent to nitrate supplementation may also contribute to faster PCr recovery in hypoxia. Indeed, increased phosphorylation potential after nitrate intake may be an indication of increased mitochondrial efficiency irrespective of the inspired F_{IO2}. However, the PCr recovery was accelerated in the H-BR relative to H-PL but not the N-BR relative to N-PL, and the PCr recovery τ was not related to the baseline muscle [PCr] or the phosphorylation potential in normoxia or hypoxia. Collectively, these findings suggest that the faster PCr recovery kinetics in H-BR may be attributed predominantly to changes in O_2 delivery. The potential contribution to this effect by altered metabolic control, as indicated by favorable changes in resting phosphorylation potential and ΔG, warrants future investigation.

Resting metabolism. Nitrate supplementation resulted in an ~9% reduction in resting muscle [PCr] irrespective of the F_{IO2} of the inspired gas. This is in contrast with previous studies that have shown no significant changes in baseline [PCr] in normoxia following nitrate supplementation (2, 17, 31, 36). Although the baseline [PCr] was reduced by 8% after nitrate supplementation in hypoxia in our previous study this did not attain statistical significance (65). In the present study the reduction in baseline [PCr] was observed in seven of eight subjects in both normoxia and hypoxia, and the baseline [PCr] was inversely correlated with the plasma [NO_2^-]. This inverse correlation was also present when the current data were combined with those of Vanhatalo et al. (65) ($r = -0.33$, $P < 0.05$, $n = 59$). It is conceivable that the elevated potential for NO production following nitrate supplementation might reduce resting muscle [PCr] synthesis via reversible *S*-nitrosation of creatine kinase (CK) (1, 18). A lower muscle [PCr] would be expected to increase the sensitivity of mitochondria to stimulation by ADP (35, 67). The lower [ADP] at rest in normoxia and hypoxia in the present study may imply that mitochondrial ATP resynthesis proceeded at the same rate with less ADP stimulation after nitrate supplementation.

The ΔG decreased (became more negative) and the phosphorylation potential ($ATP/ADP \cdot P_i$) was elevated by nitrate supplementation at rest. The elevated phosphorylation potential stemmed from reductions in both [ADP] and [P_i] while the [ATP] remained unchanged. Increased phosphorylation potential is indicative of increased proton motive force (pmf) across the mitochondrial inner membrane (5). This could occur as a result of NO-mediated reduction in proton back-leakage across the inner mitochondrial membrane (10) and thereby improved mitochondrial efficiency (P/O) as a consequence of decreased expression of mitochondrial

ADP/ATP translocase (ANT) and uncoupling protein (UCP-3) (37). The estimation of [ADP] from ^{31}P-MRS data is influenced by the measured baseline [PCr], and therefore the reduced [PCr] indirectly contributed to the increased phosphorylation potential. The CK reaction is a powerful regulator of cellular energetics (48, 56, 66) and it is plausible that NO could influence either or both ends of the bioenergetic cascade observed following dietary nitrate intake (↓PCr ↔ ↑phosphorylation potential ↔ ↑pmf ↔ ↑P/O ratio). It should be noted, however, that the reduction in baseline [PCr] that occurred alongside the decreased ΔG and increased phosphorylation potential does not imply causality. Indeed, increased muscle [PCr] subsequent to Cr supplementation has not been associated with alterations in muscle bioenergetics (ΔG) (6, 47) and CK inhibition has no effect on the mitochondrial P/O ratio at least in cardiomyocytes (20). The changes in phosphorylation potential and ΔG at rest in this study are consistent with the increased mitochondrial efficiency reported by Larsen et al. (38), and it is possible that these changes also underlie a reduction in resting metabolic rate recently reported following nitrate supplementation (38). The present data suggest that the oxidative phosphorylation system operated at a new, energetically advantageous equilibrium at a higher P/O ratio in the nitrate supplemented condition compared with placebo.

Muscle energetics during steady-state exercise. The end-exercise [PCr] (~70–77% of resting baseline) was similar to the PCr depletion of ~68% previously reported for heavy-intensity exercise (i.e., exercise performed above lactate threshold but below the critical power; 29) although a slow-component phase was only observed in five tests, all of these in hypoxia. Muscle PCr degradation and ADP accumulation were attenuated after nitrate supplementation consistent with previous findings on severe-intensity exercise in normoxia (2) and hypoxia (65). The phosphorylation potential started at a higher baseline and fell by a greater amplitude across the exercise bout in the nitrate supplemented conditions, with the phosphorylation potential remaining 58% (normoxia) and 24% (hypoxia) above the respective placebo condition at the end of 10 min of steady-state exercise. A high phosphorylation potential indicates that the ATP hydrolysis reaction equilibrium [ATP ↔ ADP + P_i (+ H^+)] is driven to the right such that it is energetically favorable to break down ATP. Likewise, the lower (more negative) ΔG in the nitrate supplemented condition compared with placebo throughout the baseline and the exercise protocol indicates greater exergonic potential of ATP hydrolysis in the nitrate-supplemented conditions.

The τ of [PCr] on-kinetics was not affected by F_{IO2} or by nitrate supplementation. The PCr on-kinetics are unchanged in the face of reduced F_{IO2} because the CK-catalyzed PCr hydrolysis at exercise onset is mainly stimulated by signals deriving from the ATPase activity at the contractile site rather than the rate of mitochondrial respiration, which is attenuated by limited O_2 availability (23). The PCr off-kinetics also appear to be impervious to NO availability in normoxia (17), but not in hypoxia (65, present study). The asymmetry between PCr on- and off-kinetics when both the F_{IO2} and NO availability are manipulated is likely a consequence of the vasodilatory effect of NO, which serves to accelerate the off-kinetics in an O_2 delivery-limited condition (F_{IO2} ~ 0.13) but has no influence under normoxic exercise and recovery in healthy subjects.

T2* signal intensity kinetics during steady-state exercise. Nitrate supplementation did not increase the amplitude of the T2* signal intensity during steady-state exercise in normoxia. This is consistent with a report of no change in the NIRS-derived amplitude of total hemoglobin (which represents a nonquantitative index of total blood volume in the muscle) in the vastus lateralis muscle during cycle ergometry after nitrate supplementation (3). A recent study by Ferguson et al. (16) showed increased blood flow in the rat hindlimb following dietary nitrate supplementation during normoxic treadmill running with blood flow being directed predominantly to muscles and muscle parts containing a high proportion of type II muscle fibers (16). The fiber-type distribution, which in human rectus femoris muscle is rather even between type I and II fiber (28), and size of the active muscle mass may contribute to the discrepancy between the present data for normoxic exercise and those of Ferguson et al. (16).

It is well-documented that hypoxic exercise engenders a considerable vasodilatory response in the active skeletal muscle which is in excess of the sum of the expected effects of hypoxia and exercise alone (8). However, nitrate supplementation did not accentuate, but rather attenuated, the T2* signal amplitude during hypoxic exercise in the rectus femoris muscle. Indeed, the T2* signal response in H-BR was not different from the normoxic conditions (Fig. 5), despite a greater circulating pool of NO_2^-, which is a potential vasodilator itself (46), and which can be reduced to NO in the vasculature (13, 14). In contrast, the T2*

signal amplitude in the H-PL condition was significantly greater relative to normoxic conditions. The tendency for attenuation of the T2* signal amplitude in H-BR may be related to either a blunted blood flow increase relative to H-PL or an increased muscle O_2 extraction, such that any increases in T2* signal intensity resulting from increased blood volume might have been offset by decreased T2* signal resulting from an increased proportion of deoxygenated blood in the muscle volume under investigation. It may be speculated that nitrate supplementation allowed the limited O_2 supply in hypoxia to be more effectively distributed to muscle fibers with a high metabolic rate, thereby increasing O_2 extraction. NO may selectively quench mitochondrial respiration in O_2 rich areas, thereby creating favorable diffusion gradients within the tissue to direct O_2 to active fibers further away from capillaries (60). In addition, increased mitochondrial efficiency (37a) and/or reduced ATP cost of force production (2, 24) following nitrate supplementation would reduce the O_2 cost at the same fixed work rate, thereby reducing blood flow requirement. However, as nitrate supplementation did not influence the T2* amplitude in normoxia, the mechanism for reduced T2* amplitude was evidently specific to the hypoxic condition only. The finding that the T2* signal amplitude did not increase after nitrate supplementation in hypoxia relative to normoxia might relate to the magnitude of exercise-induced blood flow response being related in part to relative exercise intensity (11). This interpretation is supported by the significant inverse correlation between the relative changes in T2* signal amplitude and the Q_{max} ($r = -0.60$). The Q_{max} (i.e., the maximal rate of mitochondrial ATP resynthesis) was reduced by ~24% in the H-PL condition compared with normoxia, whereas the Q_{max} in the H-BR condition was not different from that measured in normoxia. Therefore, as the external work rate was fixed in all conditions, the subjects were exercising at a higher fraction of their Q_{max} during the steady-state exercise bout in the H-PL condition, and the relatively greater exercise intensity would have necessitated a greater blood flow response. Nitrate, on the other hand, enabled the same Q_{max} to be attained in hypoxia as in the normoxic trials, and this was reflected in similar T2* signal profiles in H-BR, N-BR, and N-PL conditions.

An important consideration for the hemodynamic response during exercise is the blood flow that is directed to inactive tissue. Some vasoconstriction is necessary in inactive tissue to enable regional blood flow distribution and the maintenance of sufficient blood pressure to facilitate O_2 delivery to active tissue during exercise (4). Indeed, vasoconstriction activity was detected (as indicated by a T2* signal decrease) in the inactive left rectus femoris muscle and the right vastus medialis muscle in all experimental conditions, indicative of a metaboreflex-mediated increase in sympathetic activity. Importantly, the elevated potential for NO production following nitrate supplementation did not impede vasoconstriction in inactive muscle during exercise in normoxia or hypoxia. The similar vasoconstriction in inactive muscle and the faster T2* signal kinetics in active muscle following nitrate supplementation suggest that the reduction of circulating NO_2^- to NO was temporally and locally limited to areas of low microvascular P_{O_2}. This is consistent with the key role of NO in facilitating the matching of O_2 delivery to energy demand (63).

Blood pressure. An unexpected finding of the present study was that blood pressure was not influenced by nitrate supplementation either at rest or postexercise. This is in contrast to several studies that have reported reductions in systolic (3, 32, 36) or both systolic and diastolic blood pressure in normotensive humans (2, 31, 65, 68). The blood pressure lowering effect of dietary nitrate may be dependent on the dose and timing of supplement, as well as the baseline blood pressure (30, 68).

Technical considerations. It is important to note that the T2* weighted MR signal intensity is not a quantitative measure of muscle blood flow, and that the observed T2* signal intensity may be influenced by factors other than the inflow of oxygenated blood in the capillary network. First, the signal intensity is sensitive to alterations in the fluid distribution in the muscle and the interaction between intracellular water and muscle macromolecule concentrations. Such effects can potentially be accounted for by monitoring T2 changes in addition to T2* (15). However, simultaneous collection of T2 and T2* data would require the application of more complex scanning sequences (e.g., 57) that were not available in the present study. Second, the T2* signal intensity may also be influenced by "fresh signal sources" resulting from increased blood inflow replacing blood that has only recently been exposed to an MRI-related radiofrequency pulse and which thus manifests reduced signal intensity when exposed to a subsequent pulse as part of a dynamic scanning protocol. Given the relatively long time between pulses applied in the present study (4 s), however, any such signal saturation is considered negligible.

It is the alterations in the O_2 content of hemoglobin that give rise to the BOLD effect (69), which originates from the different magnetic properties of oxygenated and deoxygenated hemoglobin and the associated changes in the rate of MRI signal decay. An increase in BOLD signal may be the result of a decrease in deoxygenated hemoglobin (i.e., less O_2 extraction for a similar perfusion), an increase in oxygenated hemoglobin (i.e., greater blood volume or blood flow for a similar O_2 consumption), or a combination of these two scenarios within the region of interest. The magnetic fields associated with hemoglobin can potentially be long ranging, such that the BOLD-derived signal intensity alterations may occur intravascularly, resulting from the blood itself, and extravascularly due to water molecules in the surrounding tissues. However, modeling of typical capillary dimensions, hemoglobin volumes, and the effect of hemoglobin on the local magnetic field leads to the conclusion that the extravascular BOLD effect will have a minimal impact (<1%) on the total T2* MRI signal intensity (69), particularly at a low field strength such as 1.5 T used in the present study (55). There is significant debate regarding the predominant source of T2* signal changes and how this varies with the precise experimental conditions (43, 69). During an ischemic protocol (e.g., 41, 61) the BOLD effect is mainly driven by the changes in tissue perfusion upon cuff release rather than change in O_2 consumption. However, during exercise (in the absence of vascular occlusion) both blood flow and O_2 consumption change and during the postexercise recovery the kinetics of these two variables may differ to an extent that the T2* signal intensity becomes dissociated from blood flow. Therefore, the T2* may be considered to reflect perfusion-related changes in oxygenated and deoxygenated hemoglobin in the vascular compartment rather than blood flow per se during exercise and subsequent recovery (27).

Implications. It has been proposed that dietary nitrate supplementation may be particularly effective in enhancing O_2 delivery and alleviating exercise intolerance in conditions where NO production via the O_2-dependent NOS pathway is diminished as a result of ageing and/or disease (7, 31, 52). We have previously shown that in healthy subjects, dietary nitrate restores exercise tolerance in hypoxia to a similar level as measured in normoxia (65) which may have important implications for improving functional capacity and quality of life in individuals who suffer from an O_2 delivery limitation (51). The current results extend the mechanistic bases for this improved exercise tolerance by showing that nitrate supplementation increases the maximal oxidative metabolic rate and enables faster reoxygenation of tissue following exercise in moderate hypoxia. It is important to note that the experimental model in the present study does not account for complex metabolic perturbations that typically accompany disease conditions associated with impaired O_2 delivery. Our findings, however, provide a mechanism by which the nitrate-nitrite-NO pathway may facilitate improved muscle oxidative function in hypoxia. Indeed, dietary nitrate improved exercise tolerance and muscle oxygenation (estimated via NIRS) in peripheral arterial disease patients suffering from intermittent claudication (34). Further clinical trials are necessary to evaluate the translational value of dietary nitrate supplementation in disease populations.

Conclusions. This study provides new insight into the effects of dietary nitrate supplementation on skeletal muscle energetics and O_2 delivery during normoxic and hypoxic exercise and subsequent recovery. Nitrate enabled faster T2* signal intensity off-kinetics during recovery from exercise, which may be indicative of increased oxygenation and/or changes in O_2 extraction. The faster [PCr] recovery in hypoxia following nitrate supplementation was positively correlated with the τ of T2* off-kinetics, suggesting that the faster PCr resynthesis (and the higher Q_{max}) in hypoxia may result from improved O_2 availability. Another original finding of the present study was that dietary nitrate reduced resting muscle PCr content, which has implications for future investigation into the effects of dietary nitrate on respiratory control. The greater muscle phosphorylation potential and lower ΔG after nitrate supplementation are consistent with improved mitochondrial efficiency. Collectively, the present data provide compelling evidence that dietary inorganic nitrate modulates muscle energetics and O_2 delivery during hypoxic exercise in a manner that might be conducive to improved muscle function in situations where muscle O_2 delivery is impaired.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.V., A.M.J., P.G.W., and J.F. conception and design of research; A.V., J.R.B., and J.F. performed experiments; A.V., J.R.B., and J.F. analyzed data; A.V., A.M.J., J.R.B., P.G.W., and J.F. interpreted results of experiments; A.V. prepared figures; A.V. drafted manuscript; A.V., A.M.J., J.R.B., P.G.W., and J.F. edited and revised manuscript; A.V., A.M.J., J.R.B., P.G.W., and J.F. approved final version of manuscript.

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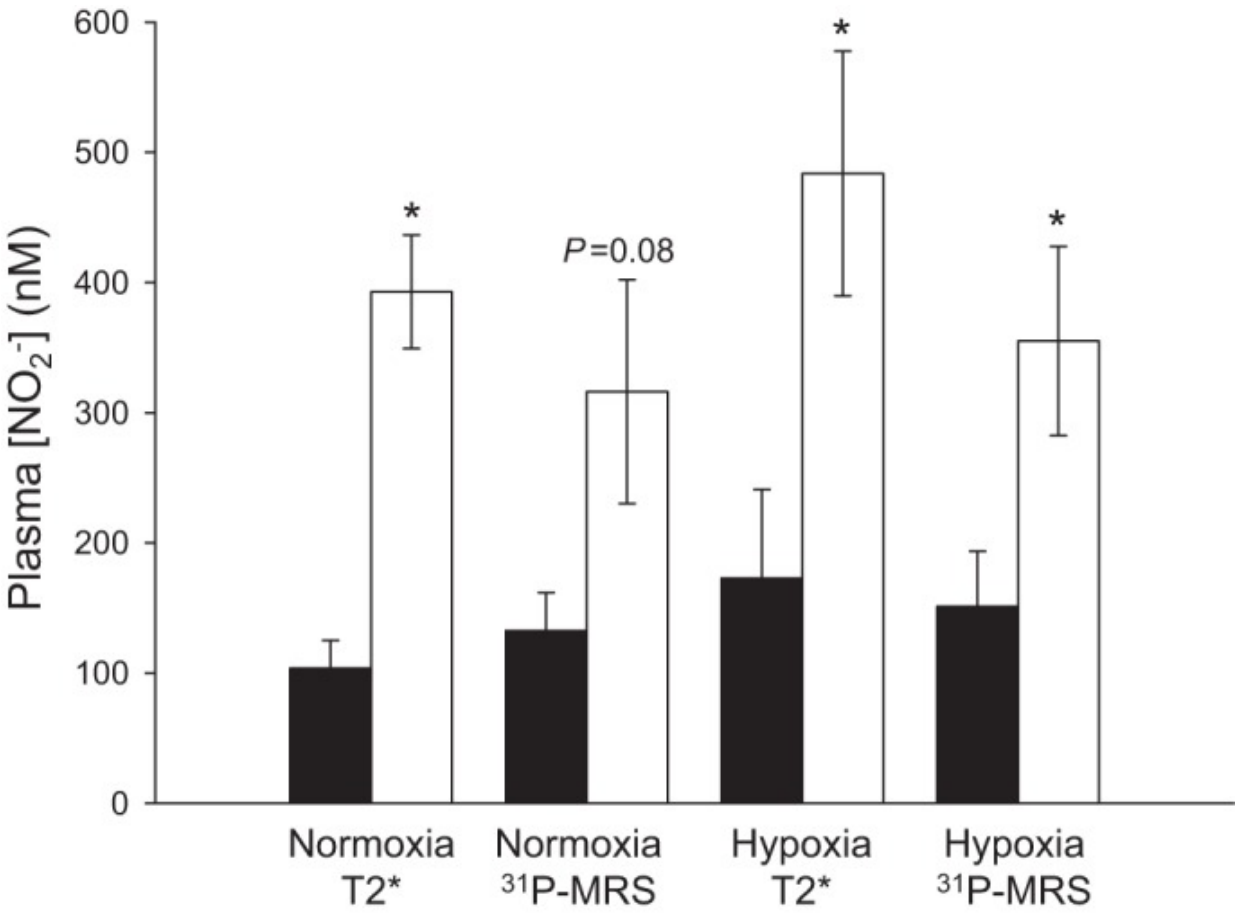
Figures and Tables

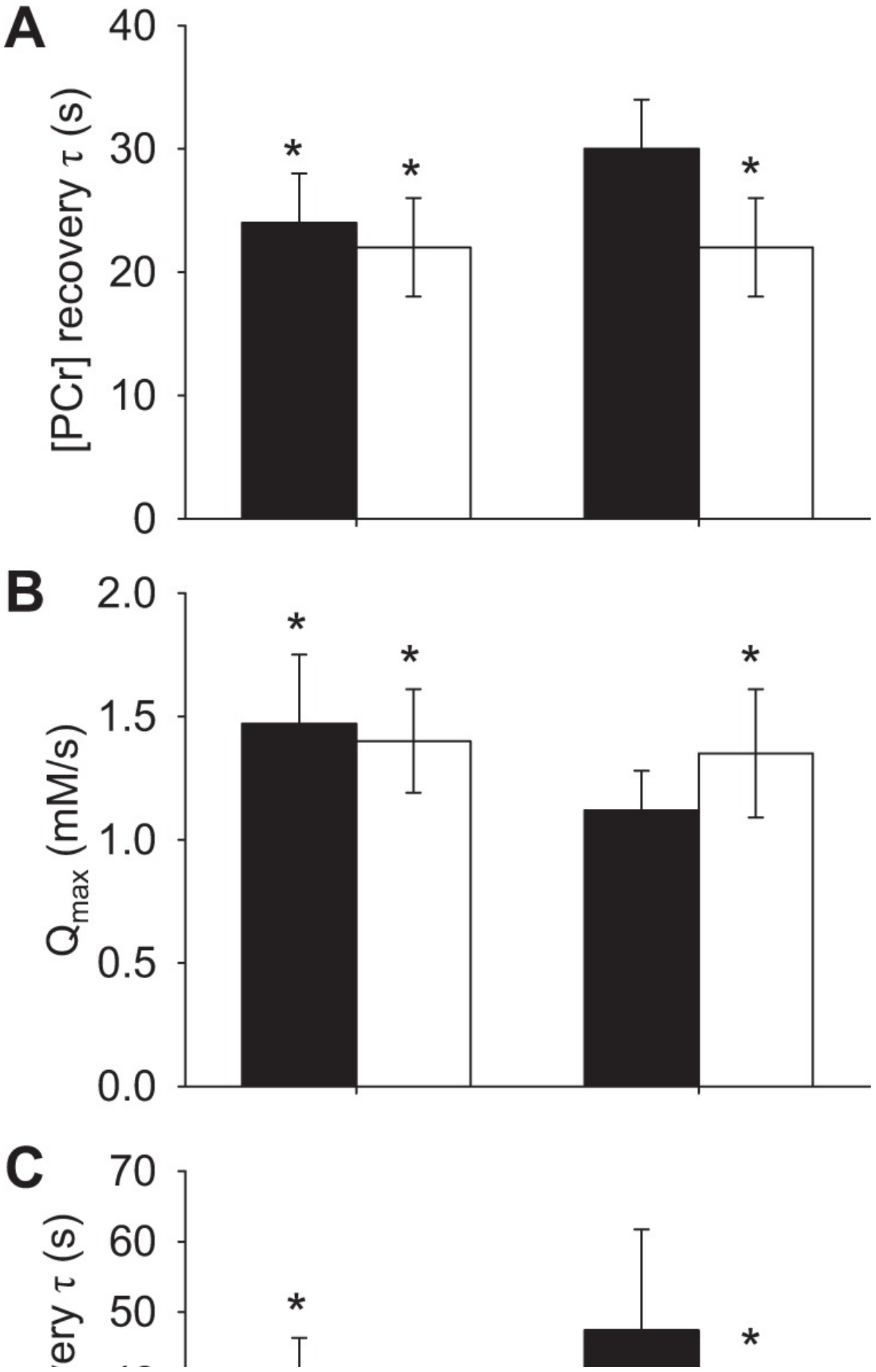
Fig. 1.

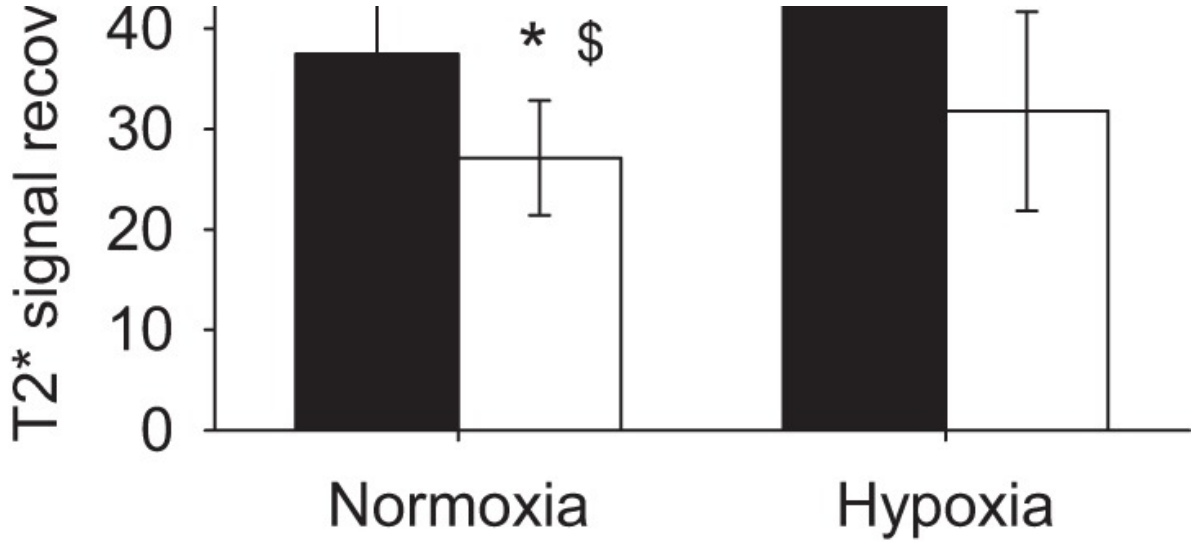


Plasma [NO_2^-] (mean ± SE) prior to normoxic and hypoxic tests was lower in placebo (PL; black bars) than beetroot juice

(BR; white bars). *Different from PL, $P < 0.05$.

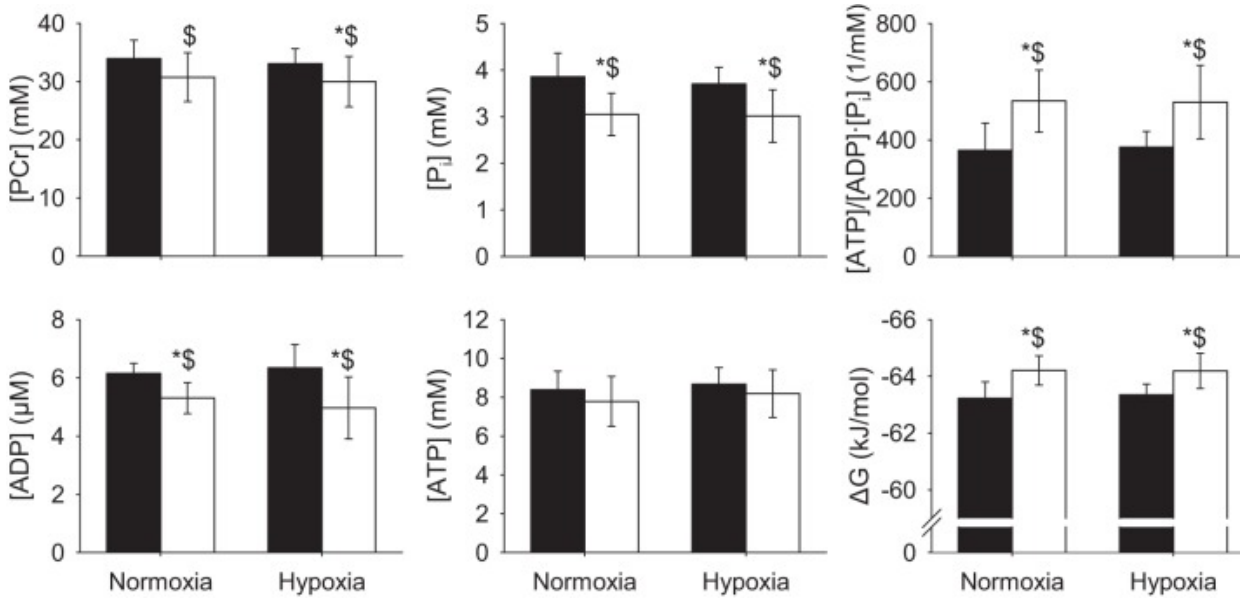
Fig. 2.





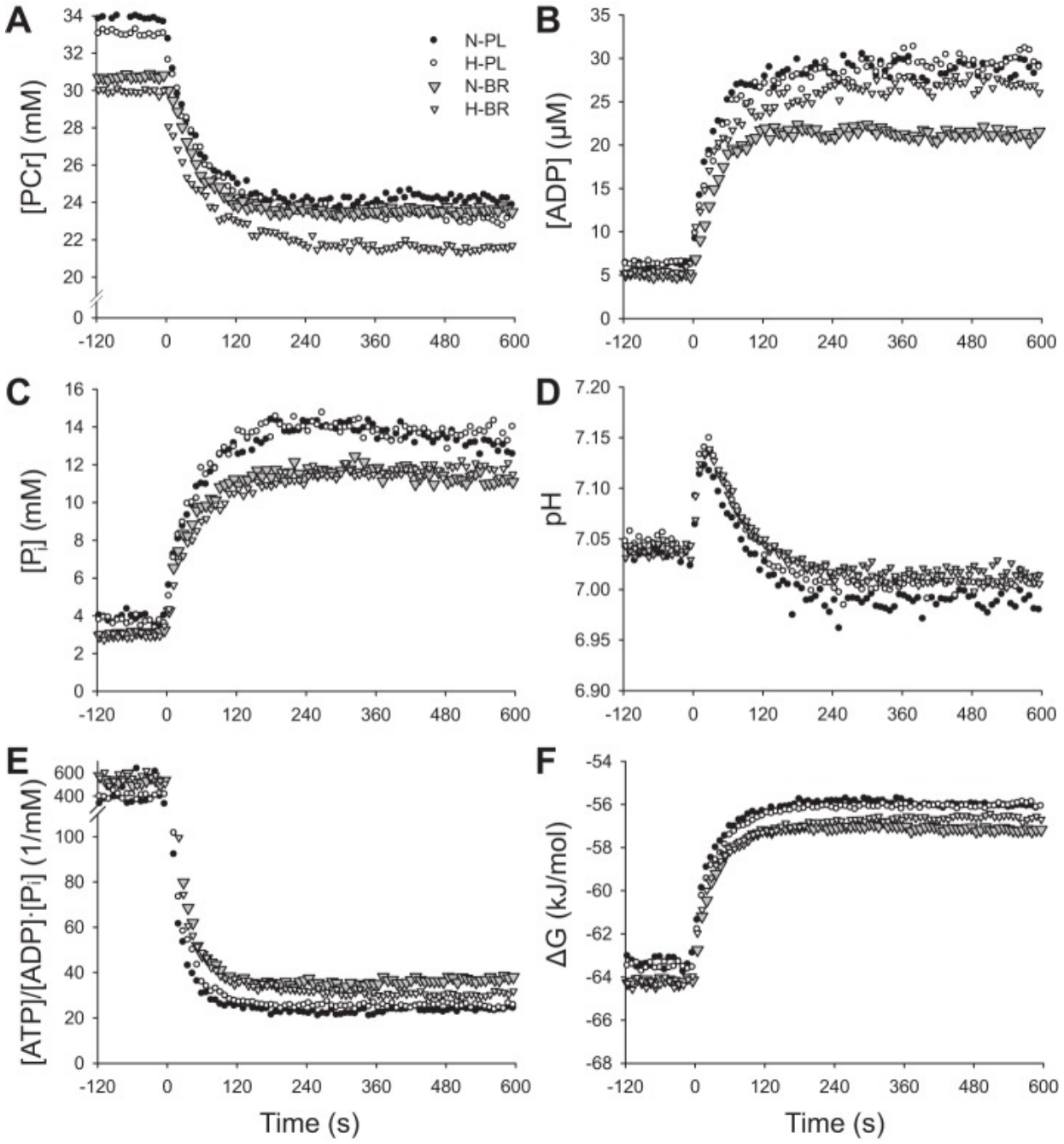
The [PCr] recovery τ (*A*), the maximal rate of muscle oxidative ATP resynthesis (Q_{max}; *B*), and the effective transverse relaxation time (T2*) recovery τ (*C*) after BR (white bars) compared with PL (black bars) supplementation. *Different from hypoxic (H)-PL, $P < 0.05$; $different from normoxic (N)-PL, $P < 0.05$.

Fig. 3.



Muscle metabolite concentrations, phosphorylation potential, and ΔG at rest. The baseline [PCr], [ADP], [P_i], and ΔG were lower and the phosphorylation potential was higher in normoxia and hypoxia after BR (white bars) compared with PL (black bars) supplementation. *Different from H-PL, $P < 0.05$; $different from N-PL, $P < 0.05$.

Fig. 4.



Muscle [PCr] (*A*), [ADP] (*B*), [P_i] (*C*), pH (*D*), phosphorylation potential (*E*), free energy of ATP hydrolysis (ΔG; *F*) during a 2-min resting baseline and 10 min of steady-state exercise. The amplitude of PCr degradation was attenuated and the amplitude of phosphorylation potential change during exercise was greater in both normoxia and hypoxia after BR supplementation compared with PL. There were no significant differences in pH between conditions. Black circles, N-PL; white circles, H-PL; gray triangles, N-BR; white triangles, H-BR.

Table 1.

Muscle metabolic responses during 10 min of steady-state exercise in normoxia and hypoxia following placebo and nitrate (BR) supplementation. Amplitude indicates the change from resting baseline to end-of-exercise

	N-PL	N-BR	H-PL	H-BR
[PCr], mM				
End-exercise	24.1 ± 2.8	23.6 ± 3.6	23.0 ± 4.9	21.6 ± 3.3[b]
Amplitude	−9.9 ± 1.4	−7.1 ± 2.0[b]	−10.0 ± 4.7	−8.3 ± 2.3[b]
τ (s) on-kinetics	56 ± 27	66 ± 26	62 ± 20	53 ± 20
[P_i], mM				

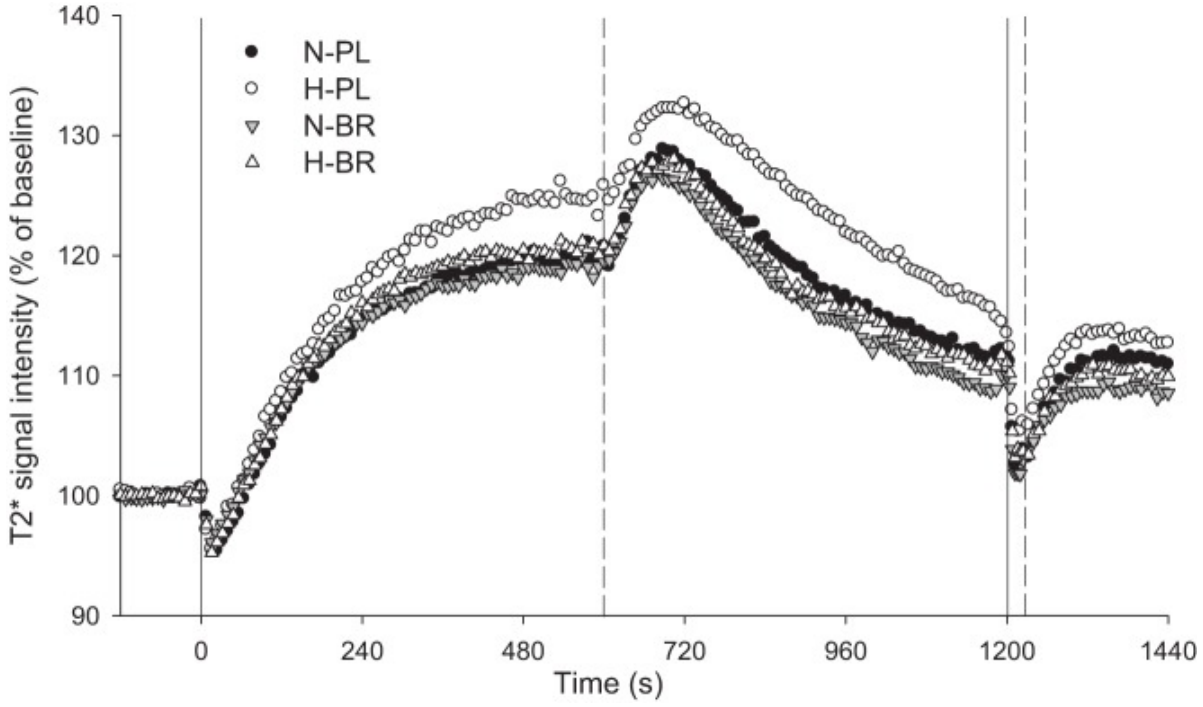
	End-exercise	12.7 ± 2.8	11.1 ± 3.0	13.5 ± 3.3	11.7 ± 4.2
	Amplitude	8.9 ± 2.6	8.1 ± 2.7	9.8 ± 3.1	8.7 ± 3.7
[ADP], μM					
	End-exercise	29.0 ± 3.5	21.1 ± 5.9[ab]	30.1 ± 8.6	26.7 ± 7.7
	Amplitude	22.9 ± 3.7	15.8 ± 5.7[b]	23.8 ± 8.8	21.7 ± 7.8
pH					
	End-exercise	6.99 ± 0.04	7.01 ± 0.04	7.01 ± 0.05	7.01 ± 0.05
	Amplitude	−0.05 ± 0.05	−0.02 ± 0.04[b]	−0.04 ± 0.04	−0.03 ± 0.05
ΔG, kJ/mol					
	End-exercise	−56.1 ± 0.7	−57.2 ± 1.1[b]	−56.0 ± 1.3	−56.6 ± 1.3
	Amplitude	7.2 ± 0.8	7.0 ± 1.0	7.5 ± 1.5	7.6 ± 0.8[b]
ATP/ADP·P_i, 1/mM					
	End-exercise	24 ± 6	38 ± 17[b]	25 ± 12	31 ± 14
	Amplitude	−340 ± 91	−496 ± 104[ab]	−350 ± 56	−499 ± 115[ab]

Values are means ± SD. Amplitude indicates the change from resting baseline to end-of-exercise.

PL, placebo; BR, beetroot juice; H, hypoxia; N, normoxia.

[a]Different from H-PL, $P < 0.05$;
[b]different from N-PL, $P < 0.05$.

Fig. 5.



The T2* signal intensity in the right rectus femoris muscle throughout the exercise protocol including 1 min of rest, 10 min of steady-state exercise, 10 min of rest, 24 s of high-intensity exercise, and 3 min 36 s of rest. The solid lines indicate the start of exercise and the dashed lines indicate the end of exercise. The amplitude of the T2* signal from baseline to end of 10 min of steady-state exercise was not different in the H-BR condition compared with N-PL and N-BR, while the amplitude in H-PL was significantly greater than in the normoxic conditions. The T2* signal recovery kinetics were modelled over the first 90 s of recovery following the 10-min and the 24-s exercise bouts.

Table 2.

Blood pressure and heart rate at resting baseline and immediately after 10 min of steady-state exercise in

normoxia and hypoxia following placebo and nitrate (BR) supplementation

		N-PL	N-BR	H-PL	H-BR
Baseline					
	BP systolic, mmHg	116 ± 13	118 ± 7	120 ± 10	119 ± 12
	BP diastolic, mmHg	70 ± 13	71 ± 7	72 ± 10	74 ± 10
	MAP, mmHg	85 ± 13	87 ± 7	88 ± 10	89 ± 11
	HR, beats/min	62 ± 8[ab]	64 ± 6[ab]	71 ± 7	71 ± 10
Postexercise					
	BP systolic, mmHg	129 ± 15	131 ± 12	130 ± 14	131 ± 15
	BP diastolic, mmHg	80 ± 10	80 ± 7	79 ± 9	78 ± 13
	MAP, mmHg	96 ± 12	97 ± 8	96 ± 10	96 ± 13
	HR, beats/min	71 ± 10[ab]	74 ± 7[ab]	82 ± 10	81 ± 13

Values are means ± SD.

HR, heart rate; MAP, mean arterial pressure.

[a]Different from H-PL, $P < 0.05$;
[b]different from H-BR, $P < 0.05$.