

**TITLE**

Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia

**AUTHORS**

Vanhatalo, A; Fulford, J; Bailey, Stephen J.; et al.

**JOURNAL**

Journal of Physiology

**DEPOSITED IN ORE**

31 October 2013

This version available at

<http://hdl.handle.net/10871/13884>

---

**COPYRIGHT AND REUSE**

Open Research Exeter makes this work available in accordance with publisher policies.

**A NOTE ON VERSIONS**

The version presented here may differ from the published version. If citing, you are advised to consult the published version for pagination, volume/issue and date of publication

# **Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia**

Anni Vanhatalo<sup>1</sup>, Jonathan Fulford<sup>2</sup>, Stephen J. Bailey<sup>1</sup>, James R. Blackwell<sup>1</sup>, Paul G. Winyard<sup>2</sup>, Andrew M. Jones<sup>1</sup>

<sup>1</sup> Sport and Health Sciences, College of Life and Environmental Sciences and <sup>2</sup> Peninsula College of Medicine and Dentistry, St. Luke's Campus, University of Exeter, Heavitree Road, Exeter, EX1 2LU, United Kingdom.

Address for Correspondence:

Anni Vanhatalo PhD

Tel: 01392 262815

Fax: 01392 264726

E-mail: a.vanhatalo@exeter.ac.uk

Running title: Dietary nitrate and exercise in hypoxia

**Key words:** nitric oxide; hypoxic vasodilatation; <sup>31</sup>P-MRS

**Word count:** 5339

**Table of Contents category:**

## Abstract

Exercise in hypoxia is associated with reduced muscle oxidative capacity and impaired exercise tolerance relative to normoxia. Nitric oxide (NO) is a key signalling molecule for hypoxic vasodilatation which may facilitate better matching of muscle perfusion to metabolic activity. The purpose of this study was to assess the effects of dietary nitrate intake (which increases plasma [nitrite] and thus NO bioavailability) on muscle metabolism and exercise tolerance in hypoxia. Nine healthy subjects (2 female; mean  $\pm$  SD age  $28 \pm 7$  years) were tested on three occasions in a single-blind, randomised crossover design. Subjects completed one trial in normoxia (21.0% O<sub>2</sub>; CON) and two trials in hypoxia (14.5% O<sub>2</sub>). During 24 h prior to the hypoxia trials, subjects consumed 0.75 L of nitrate-rich beetroot juice (9.3 mmol nitrate; H-BR) or 0.75 L of nitrate-depleted beetroot juice (0.006 mmol nitrate; H-PL). Subjects performed a series of low-intensity and high-intensity knee-extension exercise bouts for the assessment of muscle metabolism with calibrated <sup>31</sup>P-MRS and exercise tolerance (time to the limit of tolerance; T<sub>lim</sub>) and. Plasma [nitrite] was elevated ( $P < 0.01$ ) following BR ( $194 \pm 51$  nM) compared to PL ( $129 \pm 23$  nM) and CON ( $142 \pm 37$  nM). T<sub>lim</sub> was reduced in H-PL compared to CON ( $393 \pm 169$  vs.  $471 \pm 200$  s;  $P < 0.05$ ) but was not different between CON and H-BR ( $477 \pm 200$  s). The end-exercise [PCr] was not different between conditions, but the overall rate of decline in [PCr] was greater ( $P < 0.01$ ) in H-PL ( $63 \pm 28$   $\mu\text{M}\cdot\text{s}^{-1}$ ) than in CON ( $48 \pm 24$   $\mu\text{M}\cdot\text{s}^{-1}$ ) and H-BR ( $48 \pm 21$   $\mu\text{M}\cdot\text{s}^{-1}$ ). The [PCr] recovery time constant was greater ( $P < 0.01$ ) in H-PL ( $29 \pm 5$  s) compared to CON ( $23 \pm 5$  s) and H-BR ( $24 \pm 5$  s). Dietary nitrate supplementation reduced muscle metabolic perturbation during high-intensity exercise in hypoxia and restored exercise tolerance to that observed in normoxia. Nitrate supplementation also negated the slowing of [PCr] recovery kinetics which was observed in hypoxia, indicating that the O<sub>2</sub> availability was enhanced and muscle oxidative function restored to the normoxic level.

## Introduction

Hypoxia has far reaching consequences for skeletal muscle energy metabolism and fatigue development during exercise. Breathing a gas mixture with a reduced fraction of O<sub>2</sub> (F<sub>IO<sub>2</sub></sub>) results in a reduced O<sub>2</sub> partial pressure (P<sub>O<sub>2</sub></sub>) gradient between the microcirculatory and intracellular compartments, a reduction in intracellular P<sub>O<sub>2</sub></sub> (Richardson et al. 1995), and a compensatory increase in blood flow. A fixed sub-maximal work-rate is associated with the same O<sub>2</sub> uptake ( $\dot{V}_{O_2}$ ) but a greater muscle metabolic perturbation in hypoxia compared to normoxia (Adams & Welch 1980; Hogan et al. 1999; Linnarsson et al. 1974; Wilkins et al. 2006). Reduced O<sub>2</sub> availability mandates greater concentrations of other regulators of respiration in order to stimulate the mitochondria to maintain the required rate of oxidative ATP turnover; namely, ADP, P<sub>i</sub> and NADH, which are derived through elevated rates of phosphocreatine (PCr) hydrolysis and glycolysis (Hogan et al. 1999; 1983). The net result of hypoxia relative to normoxia at work rates >50% of maximum is accelerated depletion of muscle PCr and glycogen and a more rapid accumulation of fatigue-related metabolites (ADP, P<sub>i</sub>, H<sup>+</sup>) which contribute to impaired exercise tolerance (Hogan et al. 1999; Richardson et al. 1999; Allen et al. 2008). Depending on the severity of hypoxia and the mitochondrial capacity of the individual, hypoxia also attenuates the maximal oxidative metabolic rate, which is reflected in a slowing of [PCr] recovery kinetics following cessation of exercise (Blei et al. 1993; Haseler et al. 1999; Paganini et al. 1997).

The compensatory vasodilatation during hypoxic exercise is likely mediated by several synergistic factors including  $\beta$ -adrenergic and adenosine receptor activation, prostaglandin synthesis and the release of nitric oxide (NO) (Casey et al. 2010; 2011; MacLean et al. 1998). There is evidence to suggest that NO plays an increasingly important vasodilatory role independent of prostaglandins and  $\beta$ -adrenergic activation at higher metabolic rates (Casey et al. 2010; 2011; Wilkins et al. 2008). NO is released by the endothelium in response to exercise, and is also derived through the reduction of dietary inorganic nitrate. Nitrate is reduced to nitrite (NO<sub>2</sub><sup>-</sup>) and further to NO by various pathways (such as haemoglobin and xanthine oxidoreductase) which are potentiated in hypoxic and acidic conditions (Maher et al. 2008; Millar et al. 1998; Modin et al. 2001). It has been suggested that elevated NO

availability may facilitate a more precise local matching of blood flow to metabolic rate (Thomas et al. 2001), which may reduce the metabolic perturbation as exercise proceeds.

Dietary nitrate intake is associated with elevated plasma  $[\text{NO}_2^-]$  and reduced blood pressure in normotensive humans (Bailey et al. 2009; Kapil et al. 2010; Larsen et al. 2007; Vanhatalo et al. 2010; Webb et al. 2008). Nitrate supplementation has also been shown to reduce the  $\text{O}_2$  (and ATP) cost of steady-state low-intensity exercise, to reduce the rate of PCr degradation during high-intensity exercise, and to increase exercise tolerance (Bailey et al. 2009; 2010; Larsen et al. 2007). While increased NO bioavailability appears to be beneficial to cardiovascular health, reduced NO synthesis is characteristic of a number of pathologies. Aging and poor cardiovascular health are associated with uncoupling of endothelial NO-synthase (eNOS), which results in reduced capacity for endogenous NO production (Sindler et al. 2009; Yang et al. 2009). The nitrate-nitrite-NO pathway, which is not dependent on NOS function, is enhanced by acidic and hypoxic conditions extant during repeated muscle contractions and in poorly perfused muscle (Bryan 2006; van Faassen et al. 2009). Nitrate supplementation may therefore represent a potential therapeutic intervention to alleviate the effects of hypoxia on skeletal muscle metabolism and performance. This is important, because tissue hypoxia contributes to exercise intolerance in several disease conditions including peripheral arterial disease, diabetes and chronic heart failure (Bulmer & Coombes 2004; Ellis et al. 2010; Kenjale et al. 2011) as well as in exposure to high altitude.

The greater muscle metabolic perturbation and reduction in exercise tolerance that is typically observed in hypoxia compared to normoxia may be attenuated when hypoxic exercise is preceded by dietary nitrate intake. The oral administration of pharmacological sodium nitrate to human subjects is restricted under UK legislation. We therefore used nitrate-rich beetroot juice (BR; Bailey et al. 2009; Kapil et al. 2010; Webb et al. 2008) to elevate NO bioavailability prior to exercise testing in moderate normobaric hypoxia (14.5%  $\text{O}_2$  in balance  $\text{N}_2$ ). The purpose of this study was to test the hypotheses that nitrate supplementation will restore the rate of muscle metabolic perturbation, exercise tolerance and [PCr] recovery kinetics in hypoxia to the normoxic level. Specifically, we hypothesised that 1) high-intensity exercise will be associated with greater rate of changes in muscle [PCr],  $[\text{P}_i]$ , [ADP] and pH during exercise in hypoxia with placebo supplementation (H-PL) than in normoxia (control; CON) and in hypoxia following nitrate supplementation (H-BR); 2) the time-to-exhaustion ( $T_{\text{lim}}$ ) during high-intensity exercise in H-PL will be shorter than the  $T_{\text{lim}}$  in CON and H-BR;

and 3) the time constant of PCr recovery following exercise in H-PL will be greater than the recovery time constants measured in CON and H-BR.

## **Methods**

### *Ethical approval*

All procedures were approved by the University of Exeter research ethics committee and were 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*

Nine healthy subjects, who were moderately trained in recreational sport, volunteered to participate in this study (mean  $\pm$  SD: age  $28 \pm 7$  years, body mass  $73.4 \pm 12.6$  kg, height  $1.77 \pm 0.05$  m; including 2 females). Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h post-prandial, and to avoid strenuous exercise in the 24 h preceding each testing session. 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 in order to preserve commensal oral bacteria which reduce nitrate to nitrite (Govoni et al. 2008).

### *Experimental procedures*

Subjects were familiarized with the test protocol prior to data collection. During this initial visit, a high-intensity work rate which would result in exhaustion in approximately 5-8 min was determined for each subject. The following three visits (CON, H-PL, and H-BR) were allocated in a single-blind, counterbalanced, randomized order.

Exercise tests were performed in a prone position within the bore of a 1.5T superconducting magnet (Gyrosan Clinical Intera, Philips, The Netherlands) using a custom-built non-ferrous ergometer. The feet were fastened securely to padded foot braces using Velcro straps and connected to the ergometer load baskets via a rope and pulley system. Two-legged knee-extensions over a distance of  $\sim 0.22$  m were performed continuously at a constant frequency which was set in unison with the magnetic pulse sequence ( $40 \text{ pulses} \cdot \text{min}^{-1}$ ) to ensure the

quadriceps muscle was in the same phase of contraction during each MR pulse acquisition. To prevent displacement of the quadriceps relative to the MRS coil, Velcro straps were fastened over the subject's thighs, hips and lower back. The exercise protocol consisted of 4 min of low-intensity exercise and, following 6 min of passive rest, two 24-s bouts of high-intensity exercise separated by 4 min of rest. These were used for the assessment of [PCr] recovery kinetics in the absence of substantial alteration in muscle pH. After a further 6 min of rest, subjects completed one high-intensity exercise bout which was continued until the  $T_{lim}$ . Subjects were provided strong verbal encouragement to continue for as long as possible but no feedback was given on the elapsed time.  $T_{lim}$  was recorded to the nearest second. 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 aluminium load cell (Type F250EBR0HN, Novatech Measurements Ltd., St. Leonards-on-Sea, East Sussex, UK.). Work done was calculated as the product of force and displacement. The work rates were  $14 \pm 1$  W for the 4-min low-intensity bout,  $28 \pm 2$  W for the 24 s bout, and  $24 \pm 2$  W for the high-intensity bout which was continued to  $T_{lim}$ .

Subjects wore a facemask throughout all exercise tests and breathed the normoxic or hypoxic inspirate for 15 min prior to the start of the exercise protocol while resting in a prone position in the bore of the magnet. Blood pressure of the brachial artery was measured at the end of this 15 min period (Schiller Maglife Light, Siemens, Germany) and the mean value of three consecutive measurements was recorded. Heart rate and arterial  $O_2$  saturation ( $SaO_2$ ) were monitored continuously throughout each testing session with a finger probe oximeter (Nonin 7500FO, Nonin Medical Inc., Plymouth, MN). Arterial  $PO_2$  was estimated from  $SaO_2$  using the Hill equation assuming a  $P_{50}$  of 26.6 mmHg. The inspirate was generated using a Hypoxico® HYP-100 filtration system (Sporting Edge UK Ltd, Basingstoke, UK). The generator fed via an extension tube to a 150 L Douglas Bag (Cranlea & Co, 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 & Co, Birmingham, UK), which was connected to the facemask. Thus, the flow rate was maintained constant, and no re-breathing of expired air occurred.

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<sub>2</sub>, 8.0% CO<sub>2</sub> and 76.0% N gas mix (BOC Special Gases, Guildford, UK). For the normoxic CON trial, the Hypoxico® HYP-100 was switched to normobaric mode (i.e. all O<sub>2</sub> filters were inactivated such no O<sub>2</sub> was removed from ambient air), whereas during hypoxic tests, the generator was set to maximum O<sub>2</sub> filtration, which yielded an F<sub>I</sub>O<sub>2</sub> of 14.45 ± 0.05%, and an F<sub>I</sub>CO<sub>2</sub> of 0.04 ± 0.00%. The subject and the researcher running the exercise test within the MR scanner room were blinded to the inspirate being used.

### *Supplementation and nitrite analyses*

During 24 h prior to the hypoxic trials, subjects consumed 0.75 L of nitrate-rich beetroot juice containing 9.3 mmol nitrate (H-BR) or 0.75 L of nitrate-depleted beetroot juice containing 0.006 mmol nitrate (H-PL; Beet It, James White Drinks Ltd., Ipswich). Nitrate was removed from the placebo product before pasteurization by passing beetroot juice through a column containing Purolite A520E ion-exchange resin which is specific for nitrate (Lansley et al. 2011). The supplement was taken in three equal doses approximately 24 h, 12 h and 2.5 h prior to the start of the exercise test. Upon arrival at the laboratory, a venous blood sample (6 mL) was drawn from the antecubital vein into a lithium-heparin tube (Vacutainer, Becton Dickinson, New Jersey, USA). Samples were centrifuged at 4000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was subsequently extracted and immediately frozen at -80°C, for later analysis of [NO<sub>2</sub><sup>-</sup>] using a modification of the chemiluminescence technique which we have used previously (Bailey et al., 2010; Vanhatalo et al., 2010). Equipment and surfaces were regularly rinsed with ionised water to minimise contamination of samples by extraneous sources of nitrite and nitrate. Before samples were analysed for NO<sub>2</sub><sup>-</sup> content, they were thawed at room temperature and deproteinised using zinc sulfate precipitation. The deproteinised samples were then refluxed in 0.3 M sodium iodide and glacial acetic acid at room temperature and analysed for [NO<sub>2</sub><sup>-</sup>] using a Sievers nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The nitrate concentrations of diluted beetroot supplements (100- and 1000- fold) were determined by the reduction to NO in a solution of vanadium (III) chloride in hydrochloric acid. The gas-phase chemiluminescent reaction between NO and ozone was detected from the spectral emission of the electronically excited nitrogen dioxide product, by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in the nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK).



### *MRS measurements*

Absolute concentrations of muscle metabolites were established using a calibrated  $^{31}\text{P}$ -MRS technique. Spatially localized spectroscopy was undertaken prior to the exercise protocol to determine the relative signal intensities obtained from a phosphoric acid source placed within the scanner bed and an external  $\text{P}_i$  solution. A subsequent unsaturated scan was obtained comparing the signals obtained from the phosphoric acid standard and  $\text{P}_i$  in the muscle tissue, where the localized voxel sampled within the muscle was of the same dimensions and distance from the coil as the external  $\text{P}_i$  solution, allowing the calculation of muscle  $\text{P}_i$  concentration following corrections for relative coil loading. PCr and ATP concentrations were then calculated using the ratio of  $\text{P}_i/\text{PCr}$  and  $\text{P}_i/\text{ATP}$  for each individual. Fast field echo images were then acquired to determine whether the muscle was positioned correctly relative to the coil. Matching and tuning of the coil was performed and an automatic shimming protocol was then undertaken within a volume that defined the quadriceps muscle. Before and during exercise, data were acquired every 1.5 s, with a spectral width of 1,500 Hz and 1K data points. Phase cycling with four phase cycles was employed, leading to a spectrum being acquired every 6 s. The subsequent spectra were quantified via peak fitting, assuming prior knowledge, using the jMRUI (version 3) software package employing the AMARES fitting algorithm (Vanhamme et al. 1997). Spectra were fitted assuming the presence of the following peaks:  $\text{P}_i$ , phosphodiester, PCr,  $\alpha$ -ATP (2 peaks, amplitude ratio 1:1),  $\gamma$ -ATP (2 peaks, amplitude ratio 1:1), and  $\beta$ -ATP (3 peaks, amplitude ratio 1:2:1). In all cases, relative amplitudes recorded during exercise were corrected for partial saturation by obtaining a baseline spectrum before exercise with long repetition time ( $\text{TR} = 20$  s) in which the relative unsaturated peak amplitudes could be determined. Intracellular pH was calculated using the chemical shift of the  $\text{P}_i$  spectral peak relative to the PCr peak. [ADP] was calculated via knowledge of [ $\text{P}_i$ ], [PCr], and pH values, taking into account the dependency of rate constants on pH (Kemp et al. 2001).

The [PCr] recovery time constant ( $\tau$ ) 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 two time constants was calculated for each subject. Metabolite concentrations (PCr, ADP,  $\text{P}_i$  and also pH) at resting baseline were calculated as the mean over the final 120 s preceding the first exercise bout and the end-exercise values were taken as the mean values measured over the final 12 s of exercise. The rates of change during high-intensity exercise were

calculated by dividing the metabolite concentration ([PCr], [P<sub>i</sub>], [ADP] or pH) at a given time point by time. The overall rate of change was calculated as the end-exercise value divided by T<sub>lim</sub>.

### *Statistical analyses*

One-way repeated measures analyses of variance were used to assess differences across the treatments (CON, H-BR and H-PL trials) with follow-up LSD pairwise comparisons as appropriate (v15.0, SPSS Inc., Chicago, IL, USA). Statistical significance was accepted at the  $P < 0.05$  level and data are presented as mean  $\pm$  SD unless stated otherwise.

## **Results**

### *Plasma [NO<sub>2</sub><sup>-</sup>] and blood pressure*

Plasma [NO<sub>2</sub><sup>-</sup>] was elevated following supplementation with nitrate-rich beetroot juice ( $194 \pm 51$  nM) compared to placebo ( $129 \pm 23$  nM;  $P < 0.05$ ) and control ( $142 \pm 37$  nM;  $P < 0.05$ ).

The blood pressure data are summarised in Table 1. The systolic BP was lower in H-BR than in H-PL ( $P < 0.05$ ) and tended to be lower than in CON ( $P = 0.07$ ), while the diastolic BP was reduced in the H-BR condition compared to H-PL and CON ( $P < 0.05$ ). Similarly, the mean arterial pressure (MAP) was lower in H-BR compared to H-PL and tended to be lower than in CON ( $P = 0.08$ ). SaO<sub>2</sub> remained stable within each condition and was lower in the H-PL ( $91 \pm 2$  %; PO<sub>2</sub>  $61 \pm 5$  mmHg) and H-BR ( $92 \pm 1$  %; PO<sub>2</sub>  $62 \pm 6$  mmHg) trials compared to CON ( $98 \pm 1$  %; PO<sub>2</sub>  $125 \pm 37$  mmHg). Heart rate at rest was not significantly different between conditions ( $64 \pm 5$  b·min<sup>-1</sup> in CON,  $72 \pm 9$  b·min<sup>-1</sup> in H-PL and  $68 \pm 6$  b·min<sup>-1</sup> in H-BR).

### *[PCr] recovery*

The reduction in muscle [PCr] from resting baseline during the 24 s high-intensity bout was not different between conditions ( $9.8 \pm 2.1$  mM in CON,  $9.6 \pm 2.1$  mM in H-PL and  $9.3 \pm 2.9$  mM in H-BR). The end-exercise pH was not different from resting baseline ( $7.06 \pm 0.03$  in CON,  $7.09 \pm 0.04$  in H-PL and  $7.08 \pm 0.04$  in H-BR). The [PCr] $\tau$  measured during recovery was significantly greater in H-PL ( $29 \pm 5$  s) than in CON ( $23 \pm 5$  s;  $P < 0.01$ ) and H-BR ( $24 \pm 5$  s;  $P < 0.01$ ).

### *Low-intensity exercise*

The muscle metabolic responses to low-intensity exercise are presented in Table 1 and illustrated in Figure 1. The HR measured at the end of exercise was lower in CON ( $76 \pm 5$  b·min<sup>-1</sup>) than in H-PL ( $86 \pm 6$  b·min<sup>-1</sup>) and H-BR ( $84 \pm 9$  b·min<sup>-1</sup>) (both  $P < 0.05$ ). The ANOVA revealed no significant differences in the baseline or end-exercise [PCr], [P<sub>i</sub>], [ADP] or pH between conditions. However, a direct comparison (one-tailed t-test) between the [PCr] amplitude in H-PL and H-BR indicated a significant difference ( $P < 0.05$ ) (Figure 1).

### *High-intensity exercise*

During high-intensity exercise, the  $T_{lim}$  was reduced in H-PL compared to CON ( $393 \pm 169$  vs.  $471 \pm 200$  s;  $P < 0.05$ ) but was not different between CON and H-BR ( $477 \pm 200$  s). The [PCr] and [P<sub>i</sub>] profiles are illustrated in Figure 2 and the [ADP] and pH responses are shown in Figure 3. There were no significant differences in muscle metabolite concentrations or pH measured at  $T_{lim}$  in the high-intensity trial (Table 2). However, more PCr had been utilised in the H-PL trial compared to CON and H-BR after 60 s, and compared to H-BR after 120 s ( $P < 0.05$ ; Table 2). The overall rates of [PCr] degradation, [P<sub>i</sub>] accumulation and pH reduction during the entire exhaustive exercise bout were greater in H-PL than in CON and H-BR (all  $P < 0.05$ ) (Table 2). The increase in [ADP] was accelerated in H-PL compared to CON and H-BR after 60 s and compared to H-BR after 120 s (Table 2).

## **Discussion**

The principal novel finding of this study was that dietary nitrate supplementation reduced muscle metabolic perturbation during high-intensity exercise in hypoxia and restored exercise tolerance to that observed in normoxia. Nitrate supplementation also abolished the reduction in the rate of PCr recovery in hypoxia, possibly due to better NO-mediated matching of muscle O<sub>2</sub> delivery to local metabolic rate. Essentially, with nitrate supplementation it was possible to attain the same maximal oxidative rate under mild hypoxia as is normally possible in normoxia. We also showed a trend towards reduced PCr utilisation during low-intensity steady-state exercise in hypoxia following nitrate intake. The role of NO and nitrite in hypoxic signalling is well recognised. However, this is the first study to demonstrate that the deleterious effects of systemic hypoxia on muscle energetics and exercise tolerance can be ameliorated by increasing NO and nitrite availability by dietary means in humans.

### *PCr recovery kinetics*

An important finding of this study was the speeding of the PCr recovery kinetics by ~16% in the nitrate supplemented condition relative to placebo in hypoxia. The rate of recovery of intramuscular [PCr] immediately following exercise is considered to reflect the maximal rate of oxidative ATP reconstitution alone, with minimal or no contribution from glycolysis (Arnold et al. 1984; Kemp et al. 1993). Provided that the pH has not declined markedly, the  $\tau$  of the monoexponential [PCr] recovery profile is independent of the level of PCr depletion at the cessation of exercise (Thompson et al. 1995). The recovery [PCr] $\tau$  in this study was similar to values reported for moderately- to well-trained subjects in normoxia (~25 s; Haseler et al. 1999). Under normal conditions, O<sub>2</sub> delivery would not be considered limiting to maximal oxidative rate in this population. A speeding of the [PCr] recovery kinetics reflects an increase in maximal oxidative rate, and can be subsequent to factors such as increased mitochondrial mass, increased oxidative enzyme activity and/or hyperoxia (Haseler et al. 1999). Possible mechanisms underlying the observed speeding of [PCr] recovery kinetics in hypoxia following nitrate supplementation include increased mitochondrial efficiency (Larsen et al. 2010), increased bulk O<sub>2</sub> delivery and/or a better matching of local perfusion to metabolic rate (Thomas et al. 2001; Victor et al. 2009).

An improved mitochondrial efficiency following nitrate intake, reported in a recent study by Larsen et al. (2010), may allow the same maximal re-synthesis rate of ATP to be reached in hypoxia compared to the normoxic, non-supplemented condition. The mitochondrial P/O ratio was elevated by 19% in human biopsy samples after 3 days of nitrate supplementation (0.1 mmol/kg/d; Larsen et al. 2010), which may be sufficient to account for the 16% speeding in the *in vivo* [PCr] recovery  $\tau$  in the present study (using a dose of  $0.13 \pm 0.02$  mmol/kg over 24 h). However, we have previously shown that increased NO bioavailability *per se* does not speed [PCr] recovery under normoxic conditions in healthy humans following 3-6 days of nitrate intake (Bailey et al. 2010 unpublished observation; Lansley et al. 2011). Therefore, although an improved P/O ratio may contribute to the reduced [PCr] recovery  $\tau$  in hypoxia to some extent, factors related to altered muscle perfusion and O<sub>2</sub> delivery are likely to be more important.

The combination of systemic hypoxia and muscle contractions creates a powerful stimulus for compensatory vasodilatation to ensure adequate O<sub>2</sub> delivery to active muscle (Calbet et al. 2009; Casey et al., 2010). The complex interactions of numerous vasodilatory mechanisms

remain under investigation. However, it is clear that NO and nitrite represent key agents in this signalling cascade (Casey et al. 2010; 2011; Heinonen et al. 2011; Maher et al. 2008; Modin et al. 2001). Elevated NO availability, as indicated by the reduced blood pressure after nitrate intake, would increase bulk blood flow to the active muscle. The plasma [nitrite] was elevated by 50% in the nitrate-supplemented hypoxia condition compared to hypoxic placebo. In addition to liberating bioactive NO, nitrite itself is recognised as a potent vasodilator and this effect is augmented in hypoxia (Maher et al. 2008). Furthermore, NO may ensure better distribution of intramuscular and intracellular O<sub>2</sub> via inhibition of cytochrome c oxidase, such that the number of critically hypoxic loci within the muscle is reduced (Hagen et al. 2003; Victor et al. 2009; Thomas et al. 2001). When the O<sub>2</sub> availability in the mitochondrion is low, the cytochrome c oxidase is predominantly in a reduced state and NO competes with O<sub>2</sub> for binding at its heme a<sub>3</sub> site (Brown & Cooper, 1994). As a result, the available O<sub>2</sub> is redistributed away from the mitochondrion causing an attenuation of hypoxic signalling (Hagen et al. 2003; Victor et al. 2009). Therefore, NO may reduce the heterogeneity of perfusion relative to metabolic activity in skeletal by quenching the metabolic activity of fibres in the close proximity of blood supply, and facilitate improved oxygenation of the more distant fibres by increasing the O<sub>2</sub> gradient (Thomas et al. 2001). The level of hypoxia induced in this study was relatively mild such that the elevated NO availability in H-BR may have sufficiently increased the bulk blood flow and improved O<sub>2</sub> distribution within the active muscle to compensate for the reduced arterial PO<sub>2</sub> and enable the same maximal oxidative rate to be achieved as in normoxia.

#### *High-intensity exercise tolerance*

High-intensity exercise tolerance increased by ~21% with nitrate supplementation compared to placebo at a fixed work rate in hypoxia. The mechanisms responsible for this effect may include the restoration of the maximal oxidative rate to the normoxic level and reduced metabolic perturbation, both of which may be afforded by increased O<sub>2</sub> delivery (as discussed above). Effectively, the fixed work rate demanded a greater proportion of the maximal oxidative rate in the H-PL trial than in the H-BR trial. The rate of PCr degradation and the increase in [P<sub>i</sub>] were both attenuated following nitrate supplementation. The attenuation of metabolic perturbation allowed high-intensity exercise to be continued for longer before the same (limiting) intramuscular environment was attained as in the placebo and control trials (Hogan et al., 1999; Vanhatalo et al. 2010). The effect of nitrate supplementation resembles

the effect of hyperoxia on [PCr] and exercise tolerance (Vanhatalo et al. 2010), suggesting that O<sub>2</sub> availability contributed to these changes.

We have previously reported a 25% increase in exercise tolerance and attenuated PCr degradation during high-intensity exercise in normoxia following nitrate supplementation (Bailey et al. 2010). The reduction in the O<sub>2</sub> cost and the estimated ATP cost of force production during high-intensity exercise afforded by dietary nitrate (Bailey et al. 2009; 2010) point to a possibility that the O<sub>2</sub> requirement of the active muscle for the same work rate may also have been lower in the H-BR trial compared to H-PL. However, the potential synergistic effects of improved O<sub>2</sub> delivery and contractile efficiency in hypoxia did not result in an improvement in exercise tolerance beyond what has been observed in normoxia (~25%; Bailey et al. 2010). It is important to note that the calculation of the ATP turnover rate using <sup>31</sup>P-MRS data relies on the [PCr] recovery  $\tau$  (Bailey et al., 2010; Kemp et al. 2007; Lanza et al. 2006). Because changes in O<sub>2</sub> delivery are known to alter the [PCr] $\tau$  (Haseler et al. 1999; 2004; 2007), this method cannot differentiate between possible changes in contractile efficiency and O<sub>2</sub> availability. Resolution of the relative contribution of changes in O<sub>2</sub> delivery, mitochondrial and/or contractile efficiency on exercise tolerance in hypoxia await further study.

#### *Low-intensity exercise*

The capacity of the respiratory and cardiovascular system to compensate for the reduced SaO<sub>2</sub> was unlikely to be reached in this study given the relatively small active muscle mass (Calbet et al. 2009). Although acute hypoxia does not alter muscle O<sub>2</sub> consumption at a fixed low-intensity work rate, the PCr utilisation typically increases compared to normoxia (Haseler et al. 1998; Figure 1). In the present study, with relatively mild hypoxia, PCr utilisation was not significantly greater in the H-PL compared to CON trial during low-intensity exercise, but there was a tendency towards a reduction in PCr utilisation in the H-BR trial compared to H-PL (Figure 1). We have previously shown that the amplitude of [PCr] utilisation is reduced during low-intensity exercise in normoxia following nitrate supplementation (Bailey et al. 2010). The linear relationship between pulmonary O<sub>2</sub> uptake ( $\dot{V}_{O_2}$ ) and intramuscular [PCr] following nitrate supplementation (Bailey et al. 2010) implies that the reduced O<sub>2</sub> cost of exercise largely derives from the contractile apparatus. Additionally, increased O<sub>2</sub> delivery in the H-BR condition may have reduced the reliance on substrate level phosphorylation, thereby sparing muscle [PCr] and resulting in a lower steady-

state [PCr] amplitude in the H-BR trial. It should be noted that the prevalence of NO as a hypoxic vasodilator may increase with exercise intensity (Casey et al. 2010), which could explain in part why the effects of dietary nitrate were greater during high-intensity exercise compared to low-intensity exercise in this study. It may also be considered that the interplay between the redox state of cytochrome c oxidase and O<sub>2</sub> availability may be more sensitive to the manipulation of NO availability at higher exercise intensities. This is because the inhibition of cytochrome c oxidase by NO in competition with O<sub>2</sub> requires the cytochrome c oxidase to be in the reduced state, which is increasingly the case when the metabolic rate is high (Taylor & Moncada, 2009; Wilson et al., 1979).

### *Implications*

The present findings suggest that dietary nitrate may have important therapeutic applications for improving skeletal muscle energetics and functional capacity when muscle O<sub>2</sub> delivery is compromised. Skeletal muscle is frequently challenged by acute and chronic hypoxia in conditions such as exercise and exposure to high altitude, as well as in cardiovascular, pulmonary and sleep disorders. For instance, it appears that chronic exposure to altitude upregulates endogenous NO production, as evidenced by 10-fold greater NO availability in native high-altitude compared to sea-level dwellers (Erzurum et al. 2007). The reduction in the rate of substrate utilisation and fatigue development during exercise and the greater maximal oxidative rate in hypoxia afforded by nitrate supplementation in this study illustrate the therapeutic potential of dietary nitrate. This is in agreement with a recent study by Kenjale et al. (2011) who showed that dietary nitrate intake improved exercise performance in peripheral arterial disease patients. Dietary nitrate may therefore represent a powerful therapeutic intervention, which can target the metabolic limitation to maximal oxidative rate by improving mitochondrial and contractile efficiency (Bailey et al. 2010; Larsen et al. 2010) as well as the potential to enhance O<sub>2</sub> delivery and distribution within the active muscle.

### *Conclusions*

Dietary nitrate intake resulted in a 50% increase in plasma [nitrite] and a significant reduction in the mean arterial pressure, indicating greater NO bioavailability in the nitrate supplemented condition compared to placebo. A key finding of this study was that nitrate restored high-intensity exercise tolerance in hypoxia to a level which was not different from that measured during the same exercise in normoxia. This effect was accompanied by a reduction in the rate of muscle metabolic perturbation (as indicated by PCr degradation and P<sub>i</sub>

accumulation) during hypoxic exercise. We also showed that the [PCr] recovery time constant, which reflects the maximal oxidative rate and is normally slowed under hypoxia, was not different in the nitrate supplemented hypoxic condition compared to normoxic control. The restoration of the maximal oxidative rate may be attributed largely to NO- and nitrite-mediated enhancements to O<sub>2</sub> delivery and distribution within the active muscle, possibly with a small contribution from enhanced mitochondrial efficiency. These findings have implications for the development of dietary interventions to alleviate deleterious effects of systemic hypoxia on skeletal muscle energetics and exercise tolerance. Further research is warranted to identify the relative contribution by putative changes in O<sub>2</sub> delivery, mitochondrial P/O ratio and contractile efficiency on hypoxic exercise tolerance following nitrate supplementation.



## References

- Adams RP & Welch HG (1980). Oxygen uptake, acid-base status, and performance with varied inspired oxygen fractions. *J Appl Physiol* **49**, 863-868.
- Allen DG, Lamb GD & Westerblad H (2008). Skeletal muscle fatigue: cellular mechanisms. *Physiol Rev* **88**, 287-332.
- Allen JD, Stabler T, Kenjale A, Ham KL, Robbins JL, Duscha BD, Dobrosielski DA & Annex BH (2010). Plasma nitrite flux predicts exercise performance in peripheral arterial disease after 3 months of exercise training. *Free Radic Biol Med* **49**, 1138-1144.
- Arnold DL, Matthews PM & Radda GK (1984). Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of <sup>31</sup>P NMR. *Magn Reson Med* **1**, 307-315.
- Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N & Jones AM (2010). Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* **109**, 135-148.
- Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna F, Wilkerson DP, Tarr J, Benjamin N & Jones AM (2009). Dietary nitrate supplementation reduces the O<sub>2</sub> cost of sub-maximal exercise and enhances exercise tolerance in humans. *J Appl Physiol* **107**:1144-1155.
- Blei ML, Conley KE & Kushmerick MJ (1993). Separate measures of ATP utilization and recovery in human skeletal muscle. *J Physiol* **465**, 203-222.
- Brown GC & Cooper CE (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* **356**, 295-298.
- Bryan NS (2006). Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med* **41**:691-701.

Bulmer AC & Coombes JS (2004). Optimising exercise training in peripheral arterial disease. *Sports Med* **34**, 983-1003.

Calbet JA, Rådegran G, Boushel R & Saltin B (2009). On the mechanisms that limit oxygen uptake during exercise in acute and chronic hypoxia: role of muscle mass. *J Physiol* **587**, 477-490.

Casey DP, Curry TB, Wilkins BW & Joyner MJ (2011). Nitric oxide-mediated vasodilation becomes independent of beta-adrenergic receptor activation with increased intensity of hypoxic exercise. *J Appl Physiol* **110**, 687-694.

Casey DP, Madery BD, Curry TB, Eisenach JH, Wilkins BW & Joyner MJ. Nitric oxide contributes to the augmented vasodilatation during hypoxic exercise. *J Physiol* **588**, 373-385.

Ellis CG, Goldman D, Hanson M, Stephenson AH, Milkovich S, Benlamri A, Ellsworth ML & Sprague RS (2010). Defects in oxygen supply to skeletal muscle of prediabetic ZDF rats. *Am J Physiol Heart Circ Physiol* **298**, H1661-H1670.

Erzurum SC, Ghosh S, Janocha AJ, Xu W, Bauer S, Bryan NS, Tejero J, Hemann C, Hille R, Stuehr DJ, Feelisch M & Beall CM (2007). Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. *Proc Natl Acad Sci USA* **104**, 17593-17598.

Govoni M, Jansson EA, Weitzberg E & Lundberg JO (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* **19**, 333-337.

Hagen T, Taylor CT, Lam F & Moncada S (2003). Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. *Science* **302**, 1975-1978.

Haseler LJ, Hogan MC & Richardson RS (1999). Skeletal muscle phosphocreatine recovery in exercise-trained humans is dependent on O<sub>2</sub> availability. *J Appl Physiol* **86**, 2013-2018.

Haseler LJ, Lin A, Hoff J & Richardson RS (2007). Oxygen availability and PCr recovery rate in untrained human calf muscle: evidence of metabolic limitation in normoxia. *Am J Physiol Regul Integr Comp Physiol* **293**, R2046-R2051.

Haseler LJ, Lin AP & Richardson RS (2004). Skeletal muscle oxidative metabolism in sedentary humans: <sup>31</sup>P-MRS assessment of O<sub>2</sub> supply and demand limitations. *J Appl Physiol* **97**, 1077-1081.

Haseler LJ, Richardson RS, Videen JS & Hogan MC (1998). Phosphocreatine hydrolysis during submaximal exercise: the effect of FIO<sub>2</sub>. *J Appl Physiol* **85**, 1457-1463.

[Heinonen] Ilkka H, Bengt S, Jukka K, Sipilä HT, Vesa O, Pirjo N, Juhani K, Kari K & Ylva H (2011). Skeletal muscle blood flow and oxygen uptake at rest and during exercise in humans: a pet study with nitric oxide and cyclooxygenase inhibition. *Am J Physiol Heart Circ Physiol* **300**, H1510-H1517.

Hogan MC, Cox RH & Welch HG (1983). Lactate accumulation during incremental exercise with varied inspired oxygen fractions. *J Appl Physiol* **55**, 1134-1140.

Hogan MC, Richardson RS & Haseler LJ (1999). Human muscle performance and PCr hydrolysis with varied inspired oxygen fractions: a <sup>31</sup>P-MRS study. *J Appl Physiol* **86**, 1367-1373.

Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ & Ahluwalia A (2010). Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* **56**, 274-281.

Kemp GJ, Meyerspeer M & Moser E (2007). Absolute quantification of phosphorus metabolite concentrations in human muscle *in vivo* by <sup>31</sup>P MRS: a quantitative review. *NMR Biomed.* **20**, 555-565.

Kemp GJ, Roussel M, Bendahan D, Le Fur Y & Cozzone PY (2001). Interrelations of ATP synthesis and proton handling in ischaemically exercising human forearm muscle studied by <sup>31</sup>P magnetic resonance spectroscopy. *J Physiol* **535**, 901-928.

Kemp GJ, Taylor DJ & Radda GK (1993). Control of phosphocreatine resynthesis during recovery from exercise in human skeletal muscle. *NMR Biomed* **6**, 66-72.

Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim E, Kraus WE & Allen JD (2011). Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *J Appl Physiol* **110**, 1582-1591.

Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N & Jones AM (2011). Dietary nitrate supplementation reduces the O<sub>2</sub> cost of walking and running: a placebo-controlled study. *J Appl Physiol* **110**, 591-600.

Lanza IR, Wigmore DM, Befroy DE & Kent-Braun JA (2006). In vivo ATP production during free-flow and ischaemic muscle contractions in humans. *J Physiol* **577**, 353-367.

Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO & Weitzberg E (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab* **13**, 149-159.

Larsen FJ, Weitzberg E, Lundberg JO & Ekblom B (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf)* **191**, 59-66.

Linnarsson D, Karlsson J, Fagraeus L & Saltin B (1974). Muscle metabolites and oxygen deficit with exercise in hypoxia and hyperoxia. *J Appl Physiol* **36**, 399-402.

MacLean DA, Sinoway LI & Leuenberger U (1998). Systemic hypoxia elevates skeletal muscle interstitial adenosine levels in humans. *Circulation* **98**, 1990-1992.

Maher AR, Milsom AB, Gunaruwan P, Abozguia K, Ahmed I, Weaver RA, Thomas P, Ashrafian H, Born GV, James PE & Frenneaux MP (2008). Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. *Circulation* **117**, 670-677.

Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R & Blake DR (1998). Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* **427**, 225-228.

Modin A, Björne H, Herulf M, Alving K, Weitzberg E & Lundberg JO (2001). Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand* **171**, 9-16.

Paganini AT, Foley JM & Meyer RA (1997). Linear dependence of muscle phosphocreatine kinetics on oxidative capacity. *Am J Physiol* **272**, C501-C510.

Richardson RS, Grassi B, Gavin TP, Haseler LJ, Tagore K, Roca J, Wagner PD (1999). Evidence of O<sub>2</sub> supply-dependent VO<sub>2</sub> max in the exercise-trained human quadriceps. *J Appl Physiol* **86**, 1048-1053.

Richardson RS, Noyszewski EA, Kendrick KF, Leigh JS & Wagner PD (1995). Myoglobin O<sub>2</sub> desaturation during exercise. Evidence of limited O<sub>2</sub> transport. *J Clin Invest* **96**, 1916-1926.

Romer LM, Haverkamp HC, Amann M, Lovering AT, Pegelow DF & Dempsey JA (2007). Effect of acute severe hypoxia on peripheral fatigue and endurance capacity in healthy humans. *Am J Physiol Regul Integr Comp Physiol* **292**, R598-R606.

Sindler AL, Delp MD, Reyes R, Wu G & Muller-Delp JM (2009). Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. *J Physiol* **587**, 3885-3897.

Taylor CT & Moncada S (2010). Nitric oxide, cytochrome C oxidase, and the cellular response to hypoxia. *Arterioscler Thromb Vasc Biol* **30**, 643-647.

Thomas DD, Liu X, Kantrow SP & Lancaster JR Jr (2001). The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O<sub>2</sub>. *Proc Natl Acad Sci U S A* **98**, 355-360.

Thompson CH, Kemp GJ, Sanderson AL & Radda GK (1995). Skeletal muscle mitochondrial function studied by kinetic analysis of postexercise phosphocreatine resynthesis. *J Appl Physiol* **78**, 2131-2139.

van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J & Gladwin MT (2009). Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev* **29**, 683-741.

Vanhamme L, van den Boogaart A & Van Huffel S (1997). Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *J Magn Reson* **129**, 35-43.

Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, Benjamin N, Winyard PG & Jones AM (2010). Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol* **299**, R1121-R1131.

Vanhatalo A, Fulford J, DiMenna FJ & Jones AM (2010). Influence of hyperoxia on muscle metabolic responses and the power-duration relationship during severe-intensity exercise in humans: a <sup>31</sup>P magnetic resonance spectroscopy study. *Exp Physiol* **95**, 528-540.

Victor VM, Nuñez C, D'Ocón P, Taylor CT, Esplugues JV & Moncada S (2009). Regulation of oxygen distribution in tissues by endothelial nitric oxide. *Circ Res* **104**, 1178-1183.

Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ & Ahluwalia A (2008). Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* **51**, 784-790.

Wilkins BW, Pike TL, Martin EA, Curry TB, Ceridon ML & Joyner MJ (2007). Exercise intensity-dependent contribution of beta-adrenergic receptor-mediated vasodilatation in hypoxic humans. *J Physiol* **586**, 1195-1205.

Wilkins BW, Schrage WG, Liu Z, Hancock KC & Joyner MJ (2006). Systemic hypoxia and vasoconstrictor responsiveness in exercising human muscle. *J Appl Physiol* **101**, 1343–1350.

Wilson DF, Erecińska M, Drown C & Silver IA (1979). The oxygen dependence of cellular energy metabolism. *Arch Biochem Biophys* **195**, 485-493.

Yang YM, Huang A, Kaley G & Sun D (2009). eNOS uncoupling and endothelial dysfunction in aged vessels. *Am J Physiol Heart Circ Physiol* **297**, H1829-H1836.

## Figure legends

**Figure 1:** Intramuscular [PCr] relative to resting baseline illustrated as group mean (panel A; error bars excluded for clarity) and in a representative individual (panel B), during low-intensity exercise in normoxia (CON), hypoxia following placebo supplementation (H-PL) and hypoxia following nitrate supplementation (H-BR).

**Figure 2:** Group mean intramuscular [PCr] (panel A) and [ $P_i$ ] (panel B) during high-intensity exercise. The  $T_{lim}$  was significantly reduced in the H-PL trial compared to CON and H-BR (\* $P < 0.05$ ). Error bars indicate SEM at task failure. Panels C and D illustrate the [PCr] and [ $P_i$ ] responses for a representative individual.

**Figure 3:** Group mean intramuscular [ADP] and pH during high-intensity exercise. Error bars indicate SEM at task failure. \* =  $T_{lim}$  less in the H-PL trial compared to CON and H-BR ( $P < 0.05$ ).



**Table 1** Blood pressure at rest and muscle metabolic responses (mean  $\pm$  SD) during low-intensity exercise in control and in hypoxia with placebo (H-PL) and nitrate supplementation (H-BR). Amplitude indicates the change from baseline to end-of-exercise. (MAP = mean arterial pressure).

	<b>CON</b>	<b>H-PL</b>	<b>H-BR</b>
<b>BP systolic (mmHg)</b>	120 $\pm$ 6	123 $\pm$ 4	114 $\pm$ 6 <sup>#</sup>
<b>BP diastolic (mmHg)</b>	71 $\pm$ 7	74 $\pm$ 7	67 $\pm$ 7 *
<b>MAP (mmHg)</b>	86 $\pm$ 5	90 $\pm$ 5	83 $\pm$ 5 <sup>#</sup>
<b>[PCr] (mM)</b>			
<b>Baseline</b>	31.5 $\pm$ 2.6	33.6 $\pm$ 2.8	30.9 $\pm$ 3.9
<b>End-exercise</b>	25.6 $\pm$ 3.1	27.4 $\pm$ 3.0	25.2 $\pm$ 3.6
<b>Amplitude</b>	-5.9 $\pm$ 1.0	-6.2 $\pm$ 1.3	-5.7 $\pm$ 1.9
<b>[P<sub>i</sub>] (mM)</b>			
<b>Baseline</b>	4.3 $\pm$ 0.8	4.4 $\pm$ 0.6	4.1 $\pm$ 0.8
<b>End-exercise</b>	8.6 $\pm$ 1.3	9.2 $\pm$ 2.0	8.5 $\pm$ 2.6
<b>Amplitude</b>	4.3 $\pm$ 1.4	4.8 $\pm$ 2.1	4.4 $\pm$ 2.6
<b>[ADP] (<math>\mu</math>M)</b>			
<b>Baseline</b>	7.0 $\pm$ 1.3	7.1 $\pm$ 0.9	6.7 $\pm$ 1.2
<b>End-exercise</b>	19.8 $\pm$ 4.3	20.0 $\pm$ 3.4	18.8 $\pm$ 4.2
<b>Amplitude</b>	12.8 $\pm$ 4.0	12.9 $\pm$ 3.5	12.1 $\pm$ 5.0
<b>pH</b>			
<b>Baseline</b>	7.03 $\pm$ 0.04	7.06 $\pm$ 0.03	7.05 $\pm$ 0.03
<b>End-exercise</b>	7.01 $\pm$ 0.04	7.03 $\pm$ 0.03	7.03 $\pm$ 0.04
<b>Amplitude</b>	-0.02 $\pm$ 0.04	-0.03 $\pm$ 0.03	-0.02 $\pm$ 0.04

\* Different from CON and H-PL,  $P < 0.05$ ; <sup>#</sup> different from H-PL,  $P < 0.05$ .

**Table 2** Muscle metabolic responses (mean  $\pm$  SD) during high-intensity exercise in control and hypoxia after placebo (H-PL) and nitrate supplementation (H-BR). Amplitude indicates the change from baseline to task failure.

	<b>CON</b>	<b>H-PL</b>	<b>H-BR</b>
<b>[PCr] (mM)</b>			
<b>Baseline</b>	31.2 $\pm$ 2.8	33.1 $\pm$ 2.8	30.6 $\pm$ 3.9
<b><math>\Delta</math> 60 s</b>	-11.4 $\pm$ 2.7	-13.2 $\pm$ 2.7 <sup>#</sup>	-11.1 $\pm$ 2.9 *
<b><math>\Delta</math> 120 s</b>	-15.1 $\pm$ 3.1	-16.6 $\pm$ 2.2	-14.1 $\pm$ 3.0 *
<b><math>\Delta</math> 180 s</b>	-17.3 $\pm$ 3.3	-18.4 $\pm$ 2.4	-16.1 $\pm$ 3.2 *
<b>At task failure</b>	12.1 $\pm$ 2.4	12.0 $\pm$ 3.6	11.3 $\pm$ 3.3
<b><math>\Delta</math> (<math>\mu\text{M}\cdot\text{s}^{-1}</math>)</b>	-48 $\pm$ 24	-63 $\pm$ 28 <sup>#</sup>	-48 $\pm$ 21*
<b>[P<sub>i</sub>] (mM)</b>			
<b>Baseline</b>	3.2 $\pm$ 0.9	3.3 $\pm$ 0.7	3.1 $\pm$ 0.7
<b><math>\Delta</math> 60 s</b>	8.5 $\pm$ 2.5	10.4 $\pm$ 2.5 <sup>#</sup>	8.5 $\pm$ 2.1*
<b><math>\Delta</math> 120 s</b>	11.7 $\pm$ 3.9	15.0 $\pm$ 3.3 <sup>#</sup>	12.2 $\pm$ 3.0*
<b><math>\Delta</math> 180 s</b>	13.4 $\pm$ 4.0	17.1 $\pm$ 4.4 <sup>#</sup>	13.8 $\pm$ 4.3 *
<b>At task failure</b>	18.3 $\pm$ 3.3	21.4 $\pm$ 4.7	19.1 $\pm$ 5.4
<b><math>\Delta</math> (<math>\mu\text{M}\cdot\text{s}^{-1}</math>)</b>	39 $\pm$ 24	54 $\pm$ 26 <sup>#</sup>	40 $\pm$ 21 *
<b>[ADP] (<math>\mu\text{M}</math>)</b>			
<b>Baseline</b>	7.2 $\pm$ 1.4	7.6 $\pm$ 1.1	6.9 $\pm$ 1.6
<b><math>\Delta</math> 60 s</b>	34.4 $\pm$ 13.0	48.1 $\pm$ 17.4 <sup>#</sup>	33.6 $\pm$ 11.3 *
<b><math>\Delta</math> 120 s</b>	46.7 $\pm$ 15.0	59.7 $\pm$ 18.5	44.0 $\pm$ 13.6 *
<b>At task failure</b>	75.5 $\pm$ 19.9	102.9 $\pm$ 58.6	84.2 $\pm$ 28.6
<b><math>\Delta</math> (<math>\text{nM}\cdot\text{s}^{-1}</math>)</b>	177 $\pm$ 107	286 $\pm$ 177	191 $\pm$ 104
<b>pH</b>			
<b>Baseline</b>	7.01 $\pm$ 0.03	7.03 $\pm$ 0.03	7.02 $\pm$ 0.03
<b>At task failure</b>	6.88 $\pm$ 0.08	6.85 $\pm$ 0.07	6.89 $\pm$ 0.07
<b>Rate of change (<math>\text{ks}^{-1}</math>)</b>	-0.32 $\pm$ 0.29	-0.52 $\pm$ 0.24 <sup>#</sup>	-0.34 $\pm$ 0.30 *

\* Different from H-PL,  $P < 0.05$ ; <sup>#</sup> different from CON,  $P < 0.05$ .

Figure 1

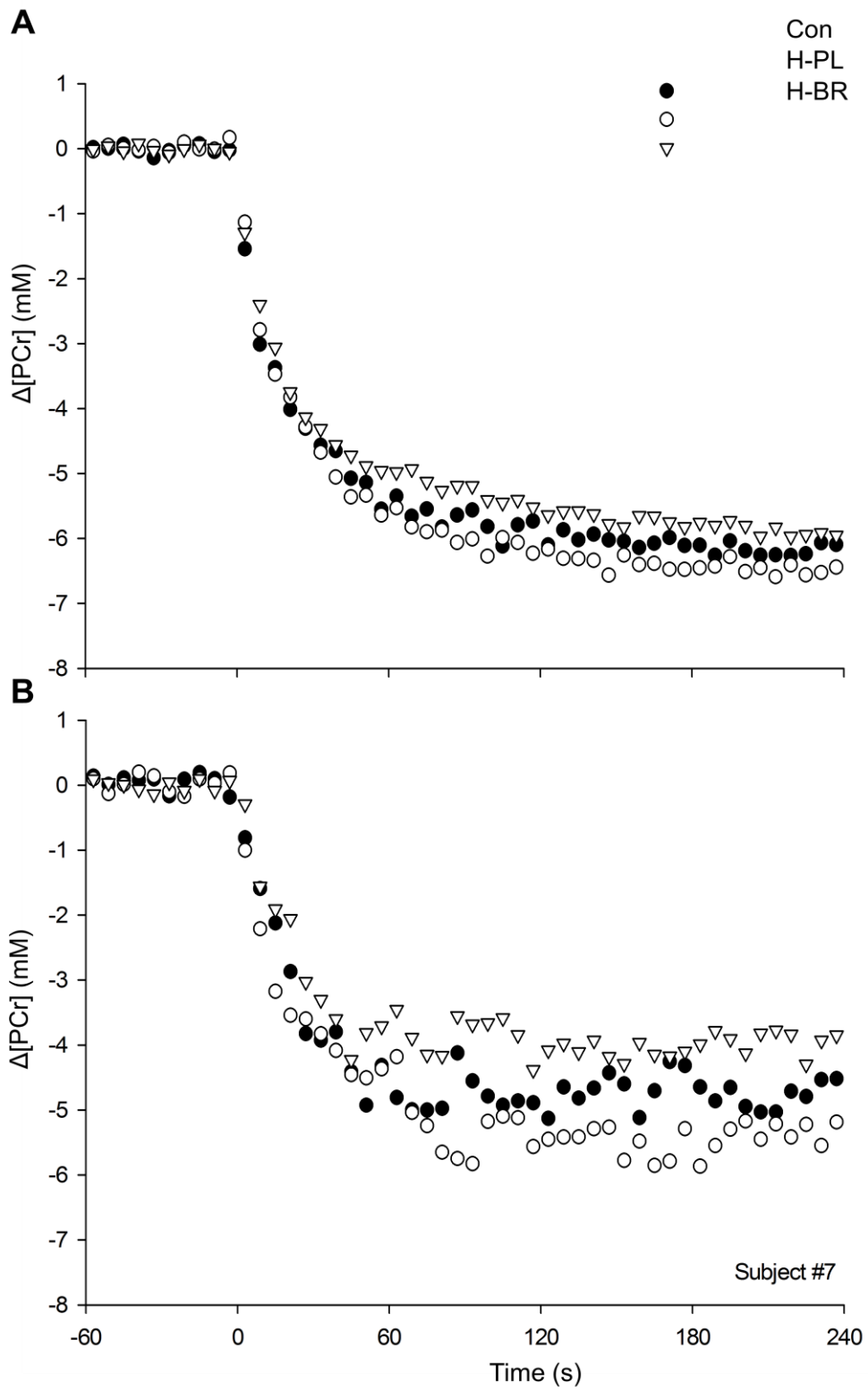


Figure 2

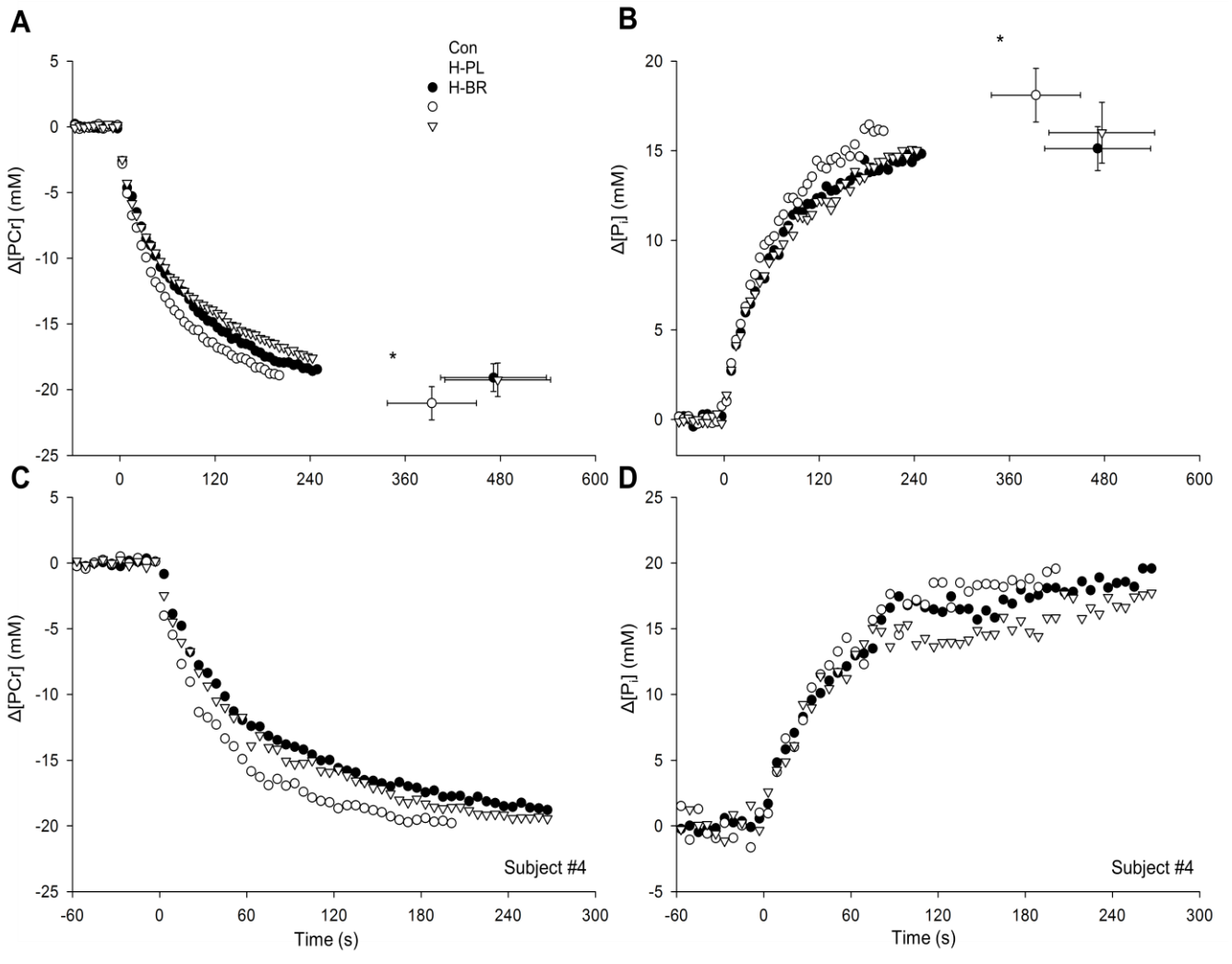


Figure 3

