Dietary nitrate supplementation: effects on plasma nitrite and pulmonary $\text{O}_2$ uptake dynamics during exercise in hypoxia and normoxia

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Dietary nitrate supplementation has been shown to increase the pulmonary uptake of oxygen ($\text{O}_2$) during submaximal exercise (4, 33, 62). NO3$^-\text{-NO}$ supplementation has been shown to reduce resting blood pressure (3, 33, 42) and oxygen uptake ($\text{V}_\text{O}_2$) during submaximal exercise (4, 39, 40, 41, 62, 67) and to improve exercise performance in young, healthy individuals exercising in normoxic conditions (14, 38). To restore sufficient $\text{O}_2$ supply, local blood flow is increased via hypoxia-induced vasodilatation with NO being implicated as a major mediator of this process (12). NO3$^-$ may also promote hypoxic vasodilatation in an NO-independent manner (16).

Dietary NO3$^-$ supplementation, in the form of nitrate salts and nitrate-rich beetroot juice (BR), represents a practical method of increasing circulating plasma [NO3$^-$] (31, 42, 67) and [NO2$^-$] (4, 33, 62). NO3$^-$ supplementation has been shown to reduce resting blood pressure (3, 33, 42) and oxygen uptake ($\text{V}_\text{O}_2$) during submaximal exercise (4, 39, 40, 41, 62, 67) and to improve exercise performance in young, healthy individuals exercising in normoxic conditions (14, 38), but not necessarily in well-trained athletes (5, 6, 66). These changes may be related to NO-mediated alterations in mitochondrial efficiency (39), muscle contractile function (3, 28), and enhanced muscle blood flow, with preferential distribution to type II fibers (23). These physiological alterations could be particularly beneficial when normal $\text{O}_2$ availability (~21%) is reduced. Indeed, NO3$^-$ supplementation in the form of BR has recently been shown to...
reduce muscle metabolic perturbation during exercise in hypoxia and to restore constant-work-rate exercise tolerance and postexercise indices of oxidative function toward values observed in normoxia (64). BR supplementation has also been shown to improve arterial and skeletal muscle oxygenation and extend incremental exercise tolerance (50), and to enhance cycling economy and time-trial performance (51) in hypoxia. However, while these studies suggest that BR can improve physiological responses and exercise performance in hypoxia, it has yet to be determined whether the effects of BR are more pronounced in hypoxia relative to normoxia.

The dose-response and pharmacodynamic relationships of BR supplementation have recently been investigated in normoxia (67), providing a guide to enable optimal timing and dosing of BR intake to elicit peak circulating plasma $[\text{NO}_2^-]$ values. However, the kinetics of plasma $[\text{NO}_2^-]$ during hypoxic exercise and subsequent recovery, and possible changes elicited by BR supplementation, are presently unknown. It was recently reported that during high-intensity, intermittent running exercise, plasma $[\text{NO}_2^-]$ declined significantly during exhaustive exercise and showed a tendency to recover back to baseline following 15 min of passive rest (68). Previous research has reported increases (1, 54) but, more commonly, decreases (6, 19, 26, 42, 63) in plasma $[\text{NO}_2^-]$ during exercise. In addition to exercise, the metabolism of NO and its derivatives are known to be influenced by intracellular PO2 and the activity being reported in hypoxia. Characterizing the kinetic changes in $[\text{NO}_2^-]$ during exercise and recovery at different intensity cycling at 75% of GET, and one bout of severe-intensity exercise for each individual were calculated, taking into account the mean response time for $\text{VO}_2$ during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the power output at GET).

Subjects

Twelve physically active male subjects (means $\pm$ SD: age, 22 $\pm$ 4 yr, height, 1.80 $\pm$ 0.06 m; body mass, 78 $\pm$ 6 kg; $\text{VO}_2\text{peak} = 58.3$ $\pm$ 6.3 ml·kg$^{-1}$·min$^{-1}$) volunteered to take part in this study. The Institutional Research Ethics Committee. All subjects gave written, fully informed consent prior to commencement of the study, once the experimental protocol, associated risks, and potential benefits of participation had been outlined. Subjects were instructed to arrive at the laboratory at least 3 h postprandial and to avoid strenuous exercise in the 24 h preceding each testing session. Subjects were asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively, and to consume the same light preexercise meal of their choice 4–5 h before testing. In addition to this, subjects were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study, since this has been shown to blunt the conversion of $\text{NO}_3^-$ to $\text{NO}_2^-$ in the oral cavity (27). Subjects were also instructed to maintain their normal dietary intake for the duration of the study. All exercise tests were performed at the same time of day (±1 h) for each subject.

Procedures

Subjects were required to attend the laboratory on six occasions over a 4-wk period. All exercise tests were performed using an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During visit 1, subjects completed a ramp incremental test to exhaustion for the determination of the maximal $\text{O}_2$ uptake ($\text{VO}_2\text{max}$) and the gas exchange threshold (GET). Subjects performed 3 min of baseline cycling at 20 W and 80 rpm, after which the power output was increased at a rate of 30 W/min in a linear fashion until volitional exhaustion. The height and configuration of the saddle and handlebars were recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged over 10-s periods. $\text{VO}_2$ was determined as the highest mean $\text{VO}_2$ during any 30-s period. The GET was determined from a number of measurements, including 1) the first disproportionate increase in $\text{CO}_2$ production ($\text{VCO}_2$) from visual inspection of individual plots of $\text{VCO}_2$ and $\text{VO}_2$; and 2) an increase in expired ventilation ($\text{V} / \text{V} / \text{O}_2$) with no increase in $\text{V} / \text{V} / \text{CO}_2$. Power outputs representing moderate- and severe-intensity exercise for each individual were calculated, taking into account the mean response time for $\text{VO}_2$ during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the power output at GET).

All subjects were familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. Visit 2 served as a familiarization to exercising in normobaric hypoxia. Following completion of the familiarization session, subjects were randomly assigned to receive 3 days of dietary supplementation with 140 ml/day of $\text{NO}_3^-$-rich BR or 140 ml/day of $\text{NO}_3^-$-depleted BR concentrate as a placebo (PL), (see Supplementation below), prior to the subsequent exercise trials.

During visits 3–6, the subjects completed step-transition, cycling exercise for the determination of pulmonary $\text{VO}_2$ and plasma $[\text{NO}_2^-]$ kinetics. In total, there were four different experimental conditions: 1) hypoxia-BR (H-BR); 2) hypoxia-PL (H-PL); 3) normoxia-BR (N-BR); and 4) normoxia-PL (N-PL). Trial order was randomly assigned in a balanced fashion, such that three subjects started on H-BR, three started on H-PL, three started on N-BR, and three started on the N-PL condition.

Upon arrival at the laboratory, a cannula (Insys-W, Becton-Dickinson, Madrid, Spain) was inserted into the subject’s antecubital vein to enable frequent blood sampling before, during, and after the exercise protocol. Prior to the exercise protocol, subjects lay in a supine position for 10 min breathing normoxic inspirate. A further 10-min period elapsed with subjects breathing either the hypoxic or normoxic inspirate. The exercise protocol involved two 5-min bouts of moderate-intensity cycling at 80% GET, and one bout of severe-intensity cycling at 75% $\Delta$ (a power output representing GET plus 75% of the difference between the power outputs at GET and $\text{VO}_2\text{peak}$) (65), which was continued to volitional exhaustion. Each
exercise bout involved an abrupt transition to the target power output initiated from a 20-W baseline, with the three exercise bouts separated by 6 min of passive recovery. The severe-intensity exercise bout was continued until task failure as a measure of exercise tolerance. The time to exhaustion was recorded when the pedal rate fell by >10 rpm below the 80 rpm pedal rate. In these bouts, the subjects were verbally encouraged to continue for as long as possible. Following exhaustion, a further 10-min recovery period elapsed with subjects continuing to breathe either the hypoxic or normoxic inspirate.

The VO2 responses for the two moderate bouts were averaged before analysis to reduce breath-to-breath noise and enhance confidence in the parameters derived from the modeling process (36). Venous blood was sampled preexercise (prior to any exercise and breathing of experimental inspirate), then during the baseline 20-W cycling preceding the first moderate transition (ModBL) and at 1 (Mod1), 3 (Mod3), and 5 (Mod5) min of the first moderate-intensity exercise bout. Further samples were drawn during the 20-W baseline preceding the severe transition (SevBL) and after 1 (Sev1) and 3 (Sev3) min of severe-intensity exercise and at exhaustion (Exh). Finally, samples were drawn during recovery from the severe bout at 1.5 (Rec1.5), 3 (Rec3), and 10 (Rec10) min.

Inspire

The inspire was generated using a Hypoxico HYP 100 filtration system (Sporting Edge UK, Basingstoke, UK), with the generator supplying the inspire via an extension conduit to a 150-litre Douglas Bag (Cranlea, Birmingham, UK). This acted as a reservoir and mixing chamber and had a separate outlet tube feeding into a two-way breathing valve system (Hans Rudolph, Cranlea). The two-way valve was connected to the mouthpiece, which provided a constant, unidirectional flow rate and ensured that no rebreathing of expired air occurred. The O2 and CO2 concentration of the inspire was monitored during each test using a Servomex 5200 High Accuracy Paramagnetic O2 and CO2 Analyzer (Servomex, Crowborough, UK). The gas analyzer was calibrated prior to each test with a 16.0% O2, 8.0% CO2, and 76.0% N2 gas mix (BOC Special Gases, Guildford, UK). For the N-PL and N-BR trials, the Hypoxico HYP-100 generator was switched to normoxic mode (i.e., all O2 filters were turned off so that no O2 was removed from the ambient air). However, during the H-PL and H-BR trials, the generator was set to maximum O2 filtration, which supplied an FIO2 of 0.131 ± 0.02, and an FICO2 of 0.004 ± 0.00.

Supplementation

After completion of the nonsupplemented visits 1 and 2, subjects were assigned in a double-blind, randomized, crossover design to receive a course of dietary NO3− placebo supplementation before visits 3–6. The supplements were either concentrated, NO3−-rich BR (2 × 70 ml/day of BR providing ~8.4 mmol NO3− per day; Beet it, James White Drinks, Ipswich, UK) or concentrated, NO3−-depleted PL (2 × 70 ml/day of PL providing ~0.006 mmol NO3− per day; Beet it, James White Drinks). The PL beverage was created by passing the juice, before pasteurization, through a column containing Purolite A520E ion-exchange resin, which selectively removes nitrate ions. The PL was identical to the BR in appearance, taste, and smell. Subjects were instructed to consume the beverages in the morning and afternoon of days 1 and 2 of supplementation, and then in the morning and 2.5 h before the exercise test on day 3. A washout period of at least 72 h separated each supplementation period. Subjects were instructed to follow their normal dietary habits throughout the testing period and to replicate their diet and timing of supplementation across conditions. Subjects were informed that the supplementation may cause beeturia (red urine) and red stools temporarily, but that this side effect was harmless.

Measurements

Venous blood samples were drawn into 5-ml lithium-heparin tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). Two-hundred microliters of blood was immediately hemolyzed in 200 µl of cold Triton X-100 buffer solution (Triton X-100; Amresco, Saloon, OH) and analyzed to determine blood [lactate] and [glucose] (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). Blood samples for the determination of plasma [NO2−] and [NO3−] were collected into lithium-heparin tubes and immediately centrifuged at 4,000 rpm and 4°C for 8 min. Plasma was extracted and immediately frozen at −80°C for later analysis of [NO2−] and [NO3−].

Prior to and regularly during analysis, all glassware, utensils, and surfaces were rinsed with deionized water to remove any residual NO2−. Plasma [NO2−] and [NO3−] were analyzed using gas phase chemiluminescence. This initially required NO2− and NO3− to be reduced to NO gas. For reduction of NO2−, undiluted plasma was injected into a glass purge vessel containing 5 ml of glacial acetic acid and 1 ml NaI solution. For NO3− reduction, plasma samples were deproteinized in an aqueous solution of zinc sulfate (10% wt/vol) and 1 M sodium hydroxide, prior to reduction to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% wt/vol). Quantification of NO was enabled by the detection of light emitted during the production of nitrogen dioxide formed upon reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyzer (Sievers NOA 280i; Analytix, Durham, UK). The concentrations of NO2− and NO3− were determined by plotting signal area (mV) against a calibration plot of 25 nM to 1 µM sodium nitrite and 100 nM to 10 µM sodium nitrate, respectively. The rate of change in plasma [NO3−] during the severe exercise bout was calculated as the difference between preexercise baseline and exercise [NO3−] values relative to exercise duration.

During all laboratory exercise tests, pulmonary gas exchange and ventilation were measured continuously with subjects wearing a nose clip and breathing through a mouthpiece and impeller turbine assembly (Triple V; Jaeger, Höchburg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O2) and infrared (CO2) analyzers (Oxycon Pro, Jaeger, Hoechburg, Germany) via a capillary line connected to the mouthpiece. Pulmonary gas exchange variables were calculated and displayed breath-by-breath. Heart rate (HR) and arterial oxygen saturation (SaO2) were continuously measured during the test protocol using a pulse oximeter device (Rad-87; Masimo, Irvine, CA), which was attached to the subject’s right index finger.

The oxygenation status of the musculus vastus lateralis of the right leg was monitored via near-infrared spectroscopy (NIRS) (NIRO 200; Hamamatsu Photonics KK, Hamamatsu City, Japan) during the exercise protocol, as described previously (4). Deoxyhemoglobin concentration ([HHb]), oxyhemoglobin concentration ([HbO2]), tissue oxygenation index (TOI) were measured.

Data Analysis

The breath-by-breath V̇O2 data from each exercise test were initially examined to exclude errant breaths caused by coughing and swallowing. Data with those values lying more than four SD from the local mean being removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values, and, for each individual, identical moderate-intensity repetitions were time-aligned to the start of exercise and ensemble-averaged. This approach enhances the signal-to-noise ratio and improves confidence in the parameters derived from the modeling process. The first 20 s of data after the onset of exercise (the phase I response) were deleted, and a nonlinear least squares algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the phase II V̇O2
responses to both moderate- and severe-intensity exercise, as described in the following equation:

$$V\dot{O}_2(t) = \bar{V}\dot{O}_2 + A_p \left[ 1 - e^{-(t/TD_{\text{p}})} \right]$$

(1)

where $V\dot{O}_2(t)$ represents the absolute $V\dot{O}_2$ at a given time $t$, $\bar{V}\dot{O}_2$ represents the mean $V\dot{O}_2$ over the final 60 s of baseline cycling; $A_p$, $TD_{\text{p}}$, and $\tau_p$ represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in $V\dot{O}_2$ above baseline. An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. The end-exercise $V\dot{O}_2$ was defined as the mean $V\dot{O}_2$ measured over the final 30 s of exercise.

The fitting strategy was subsequently used to identify the onset of any “slow component” in the $V\dot{O}_2$ response to severe-intensity exercise, as previously described (56). The fitting window was lengthened iteratively until the exponential model fit demonstrated a discernible departure from the measured response profile. Identification, via visual inspection, of the flat residual plot profile (signifying a good fit to measured data) systematically differing from zero, gave indication of the delayed slow-component onset. The magnitude of the slow component for $V\dot{O}_2$ was measured as the difference between the phase II steady-state amplitude and the final $V\dot{O}_2$ value, averaged over the last 30 s of exercise.

To obtain information on muscle oxygenation, the [HbO$_2$] response to exercise was also modeled, as described previously (4). The [HbO$_2$] kinetics for moderate- and severe-intensity exercise were determined using a single-exponential model similar to that described above (Eq. 1), with the exception that the fitting window commenced at the time at which the [HbO$_2$] signal increased 1 SD above the baseline mean (18). For moderate-intensity exercise, the fitting window was constrained to the point at which monoexponentiality became distorted, consequent to a gradual fall in [HbO$_2$], as determined by visual inspection of the residual plots. For severe-intensity exercise, the [HbO$_2$] fast and slow-phase responses were determined as described above for the $V\dot{O}_2$. The [HbO$_2$], [Hb$_{at}$], and TOI responses were not modeled as they do not approximate an exponential. Rather, the changes in these variables were assessed by determining the [HbO$_2$], [Hb$_{at}$], and TOI at baseline (60 s preceding step transition), at 120 s and at end-exercise during moderate exercise and at baseline, 60 s, 120 s, and exhaustion for severe exercise.

Statistical Analyses

Differences in the cardiorespiratory, NIRS-derived, pulse-oximetry and exercise tolerance variables between conditions were analyzed using two-way (condition × time) repeated-measures ANOVA. Blood metabolites were analyzed via two-way (condition × time) repeated-measures ANOVA, during moderate-intensity exercise, severe-intensity exercise, and recovery from exercise (condition refers to H-BR, H-PL, N-BR, or N-PL). Significant effects were further explored using simple contrasts with Fisher’s least significant difference test. One-tailed paired $t$-tests were used to compare differences in exercise tolerance between BR and PL treatments in hypoxia and normoxia. Correlations between physiological and performance variables were assessed via Pearson’s product-moment correlation coefficient. All data are presented as means ± SD with statistical significance being accepted when $P < 0.05$.  

RESULTS

Self-reported compliance to the supplementation regimen was 100% and the subjects’ food diaries confirmed that the timing of supplement taken on the morning of the laboratory tests was consistent across the experimental conditions. No deleterious side effects were reported.

Plasma [NO$_2^-$] and [NO$_3^-$]

Preexercise, plasma [NO$_2^-$] was significantly elevated in H-BR compared with H-PL (H-BR: 301 ± 89 vs. H-PL: 88 ± 56 nM; $P = 0.02$) and N-BR relative to N-PL (N-BR: 401 ± 276 vs. N-PL: 61 ± 28 nM; $P = 0.01$) but did not differ between H-BR and N-BR ($P = 0.54$) or H-PL and N-PL ($P = 0.66$). The group mean kinetic profiles of plasma [NO$_2^-$] during moderate-intensity and severe-intensity exercise and subsequent recovery are presented in Fig. 1.

Plasma [NO$_3^-$] was significantly elevated at all time points following BR compared with PL in both hypoxia and normoxia, although no differences were evident in the kinetic response during exercise and recovery (data not shown).

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Fig. 1. Plasma [NO$_2^-$] response during moderate-intensity and severe-intensity exercise and recovery following beetroot juice (BR) and placebo (PL) supplementation in normoxia (N) and hypoxia (H). Group means ± SE plasma. H-BR was greater than H-PL at each time point, and N-BR was greater than N-PL at each time point. $*P < 0.05$ for N-BR compared with H-BR. $^{b}P < 0.05$ compared with moderate baseline. $^{c}P < 0.05$ compared with severe baseline. Where error bars are not visible, the size of the data point exceeds the error.
**Moderate exercise.** ANOVA revealed there were significant main effects by condition and time on plasma [NO$_2^-$] during moderate-intensity exercise. BR supplementation significantly elevated plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma [NO$_2^-$] was significantly decreased after 5 min of moderate-intensity exercise (Mod5) compared with ModBL (ModBL: 332 ± 184 vs. Mod5: 290 ± 207 nM; $P = 0.04$). However, the decrease in plasma [NO$_2^-$] in H-BR only showed a trend toward a reduction (ModBL: 306 ± 109 vs. Mod5: 270 ± 125 nM; $P = 0.10$). The rate of decline in plasma [NO$_2^-$] from ModBL to Mod5 was not significantly different in H-BR (−7 ± 12 nM/min) compared with N-BR (−11 ± 16 nM/min), H-PL (−4 ± 6 nM/min) compared with N-PL (−2 ± 4 nM/min), H-BR (−7 ± 12 nM/min) compared with N-PL (−2 ± 4 nM/min) or N-BR (−11 ± 16 nM/min) compared with N-PL (−2 ± 4 nM/min).

**Severe exercise.** There were significant main effects by condition and time and an interaction effect for plasma [NO$_2^-$] during severe-intensity exercise to exhaustion. BR supplementation significantly elevated plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma [NO$_2^-$] significantly decreased after 3 min of severe-intensity exercise (Sev3) and at exhaustion, compared with SevBL (SevBL: 271 ± 177; Sev3: 206 ± 129; $P = 0.01$; exhaustion: 132 ± 117 nM; $P < 0.01$). In H-BR, plasma [NO$_2^-$] decreased from SevBL (277 ± 142 nM) to Sev1 (229 ± 123 nM; $P = 0.01$), Sev3 ($n = 10$; 164 ± 64 nM; $P = 0.03$) and exhaustion (171 ± 115 nM; $P < 0.01$). The absolute decline in plasma [NO$_2^-$] from SevBL to exhaustion showed a trend toward being smaller in H-BR (106 ± 60 nM) compared with N-BR (138 ± 79 nM; $P = 0.10$). In N-PL, plasma [NO$_2^-$] decreased from SevBL (40 ± 23 nM) to exhaustion (22 ± 19 nM; $P = 0.02$). This decrease was not significant in H-PL (SevBL: 53 ± 65 vs. exhaustion: 37 ± 45 nM/min; $P = 0.52$). The rate of decline in plasma [NO$_2^-$] was significantly greater from SevBL to exhaustion in H-BR compared with H-PL (H-BR: −30 ± 22 vs. H-PL: −7 ± 10 nM/min; $P < 0.01$) and in N-BR compared with N-PL (N-BR: −26 ± 19 vs. N-PL: −1 ± 6 nM/min; $P < 0.01$) but was not different between N-BR and H-BR ($P = 0.66$) or N-PL and H-PL ($P = 0.13$) (Fig. 1).

**Recovery.** During the 10-min recovery from exhaustive exercise, ANOVA revealed significant main effects by condition and time and an interaction effect for plasma [NO$_2^-$] (Fig. 1). BR supplementation significantly elevated plasma [NO$_2^-$] across all time points compared with PL in both hypoxic and normoxic conditions (all $P < 0.05$). In N-BR, plasma [NO$_2^-$] was lower at exhaustion compared with 3 min into the recovery period ($P = 0.05$), with a significant difference also evident between Rec1.5 and Rec3 ($P = 0.01$). Plasma [NO$_2^-$] was significantly higher in H-BR compared with N-BR at Rec1.5 ($P = 0.04$). In N-PL, recovery of plasma [NO$_2^-$] was evident between exhaustion and Rec10 ($P = 0.04$), with a significant increase in [NO$_2^-$] from Rec3 to Rec10 also evident ($P = 0.04$). In H-PL, plasma [NO$_2^-$] tended to recover between Rec1.5 and Rec3 ($P = 0.06$), with a further increase evident between Rec3 and Rec10 ($P < 0.01$).

Blood [glucose] was significantly reduced in H-BR compared with N-BR at Rec1.5 (H-BR: 4.3 ± 1.0 mM vs. N-BR: 5.5 ± 1.2 mM; $P = 0.01$), Rec3 (H-BR: 4.5 ± 1.1 mM vs. N-BR: 5.6 ± 1.3 mM; $P = 0.02$) and Rec10 (H-BR: 4.7 ± 1.0 mM vs. N-BR: 5.3 ± 1.0 mM; $P = 0.03$). No differences were evident between PL and BR conditions.

**Arterial O$_2$ saturation and heart rate.** The $\text{SaO}_2$ data at rest and during moderate-intensity and severe-intensity exercise are reported in Table 1. Resting $\text{SaO}_2$ and HR prior to the administration of inspirate were not significantly different between conditions. However, ANOVA revealed a significant main effect by FiO$_2$ following 10 min of breathing the hypoxic or normoxic inspirate, with $\text{SaO}_2$ being significantly reduced in H-PL compared with N-PL ($P < 0.01$) and H-BR compared with N-BR ($P < 0.01$). HR was significantly elevated in H-PL compared with N-PL ($P < 0.01$) and H-BR compared with N-BR ($P = 0.02$) in the final 30 s of gas inspiration.

**Moderate exercise.** During moderate-intensity exercise, $\text{SaO}_2$ was significantly reduced in both hypoxic conditions compared with the normoxic conditions (both $P < 0.01$) (Table 1). HR was significantly elevated in both hypoxic conditions compared with the normoxic conditions in the final 30 s of exercise (both $P < 0.01$), with H-BR being lower than H-PL ($P = 0.05$) over the entire 6-min duration.

**Severe exercise.** $\text{SaO}_2$ was significantly lower in H-PL compared with N-PL ($P < 0.01$) and in H-BR compared with N-BR ($P < 0.01$) at exhaustion following severe-intensity exercise. There were no differences in $\text{SaO}_2$ between BR and PL.

### Table 1. Arterial oxygen saturation and heart rate during rest and in response to moderate- and severe-intensity exercise

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<td>$\text{SaO}_2$, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>98 ± 2</td>
<td>97 ± 3</td>
<td>86 ± 4</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>End</td>
<td>94 ± 4</td>
<td>94 ± 4</td>
<td>80 ± 3‡</td>
<td>80 ± 4*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>97 ± 9</td>
<td>103 ± 12</td>
<td>113 ± 9</td>
<td>114 ± 12</td>
</tr>
<tr>
<td>Baseline</td>
<td>179 ± 4</td>
<td>180 ± 5</td>
<td>172 ± 6</td>
<td>171 ± 6</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. HR, heart rate; N-PL, normoxia placebo; N-BR, normoxia beetroot juice; H-PL, hypoxia placebo (NO$_2^-$-depleted beetroot juice); H-BR, hypoxia beetroot juice. *P < 0.05 compared to H-PL. †P < 0.05 compared to N-BR. ‡P < 0.05 compared to N-PL.
PL in either hypoxia or normoxia. Also, there were no differences in HR between conditions (Table 1).

\( \dot{V}O_2 \) Kinetics

Pulmonary \( \dot{V}O_2 \) responses across the four experimental conditions are presented in Figs. 2 and 3, and the parameters derived from the model fits are summarized in Table 2.

Moderate exercise. ANOVA revealed a significant main effect by supplement and an interaction effect on the \( \dot{V}O_2 \) response to moderate-intensity exercise. The \( \dot{V}O_2 \) in the final 30 s of exercise in H-BR was significantly lower compared with H-PL (\( P = 0.02 \)) and N-PL (\( P = 0.01 \)). BR supplementation also resulted in a reduced \( \dot{V}O_2 \) during baseline (20 W) exercise in hypoxia compared with PL (\( P = 0.02 \)). The \( \dot{V}O_2 \) phase II \( \tau \) tended to be increased (i.e., slower kinetics) in hypoxia compared with normoxia (\( P = 0.07 \)). Post hoc analyses revealed that the \( \dot{V}O_2 \) phase II \( \tau \) was smaller (i.e., faster kinetics) in H-BR compared with H-PL (\( P = 0.04 \)).

Severe exercise. During severe-intensity exercise, the \( \dot{V}O_2 \) slow-component amplitude (\( P < 0.01 \)) and \( \dot{V}O_2 \) at exhaustion (\( P < 0.01 \)) were significantly reduced as a result of the hypoxic inspirate in both PL and BR (Table 2). In hypoxia, BR tended to further reduce the end-exercise \( \dot{V}O_2 \) compared with H-PL (\( P = 0.07 \)), while BR had no effect upon end-exercise \( \dot{V}O_2 \) in normoxia.

NIRS

The [HHb], [HbO2], [Hbtot], and TOI values measured during moderate- and severe-intensity exercise are shown in Table 3.

![Fig. 2. Pulmonary O2 uptake (\( \dot{V}O_2 \)) responses during a step increment to a moderate-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles (●), with the PL responses being shown as open circles (○). The dashed vertical line denotes the abrupt “step” transition from baseline to moderate-intensity cycling exercise. Error bars indicate the SE. A: group mean response to moderate-intensity exercise in normoxia (−21% FIO2). B: group mean response to moderate-intensity exercise in hypoxia (−13.2 FIO2). *\( P < 0.05 \) compared with H-PL.](http://ajpregu.physiology.org/ by 10.220.33.2 on July 1, 2017)
Fig. 3. Pulmonary O2 uptake (VO2) responses and time-to-exhaustion during a step increment to a severe-intensity work rate, following PL and BR supplementation. Responses following BR are represented as solid circles (●), with the PL responses being shown as open circles (○). The dashed vertical line denotes the abrupt step transition from baseline to severe-intensity cycling exercise. Error bars indicate the SE. A: group mean response to severe-intensity exercise in normoxia (~21% FIO2). B: group mean response to severe-intensity exercise in hypoxia (~13.2% FIO2). *Time to exhaustion greater in H-BR compared with H-PL (P < 0.05; one-tailed t-test).

P = 0.50). The increase in severe-intensity exercise tolerance was correlated with the reduction in moderate steady-state VO2 following BR supplementation in hypoxia (r = −0.96; P < 0.01).

DISCUSSION

Consistent with previous findings, the decline of plasma [NO2−] during exercise was greater following BR compared with PL supplementation. However, in contrast to our experimental hypothesis, the decline of plasma [NO2−] during exercise was similar or slightly smaller in hypoxia compared with normoxia. Nonetheless, 3 days of BR supplementation significantly speeded VO2 kinetics and lowered the steady-state VO2 during moderate-intensity cycle exercise in hypoxia, but not normoxia. Furthermore, BR supplementation improved severe-intensity exercise tolerance in hypoxia (P < 0.05), but not normoxia (P > 0.05). These findings suggest that BR is more effective at improving exercise economy and exercise tolerance in hypoxia than normoxia.

Effects of BR Supplementation on the Kinetic Profile of Plasma [NO2−]

Plasma [NO2−] increased significantly following BR supplementation compared with PL, at rest and prior to administration of the inspirate. These findings are consistent with previous research, which has consistently reported elevations in plasma [NO2−] (3, 4, 33, 34, 51, 62, 67), following BR supplementation.

Previous studies have suggested that baseline plasma [NO2−] and/or the change in the concentrations of this metabolite during exercise may be associated with exercise performance (19, 53, 61, 68). This study is the first to characterize [NO2−] dynamics during and following exercise of different intensities in hypoxia and normoxia with and without NO3− supplementation. The results suggest that the metabolism of NO and its derivatives are altered by exercise and NO3− supplementation and, to a lesser extent, FIO2. The interpretation of these data is not straightforward, however. NO3− can be reduced in vivo to bioactive NO2 and further to NO (47), and this reduction of NO2 to NO is expected to be facilitated in hypoxia (13). However, NO2− is also an oxidation product of NO generation via the NOS pathway (30) with plasma [NO2−] providing a sensitive marker of NO production through NOS (43). Therefore, the dynamics of plasma [NO2−] over the exercise bouts is

Table 2. Pulmonary oxygen uptake kinetics in response to moderate- and severe-intensity exercise in hypoxic and normoxic conditions

<table>
<thead>
<tr>
<th></th>
<th>N-PL</th>
<th>N-BR</th>
<th>H-PL</th>
<th>H-BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate-intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2, ml/min</td>
<td>3500 ± 100</td>
<td>4000 ± 150</td>
<td>4500 ± 200</td>
<td>5000 ± 250</td>
</tr>
<tr>
<td>End exercise</td>
<td>1000 ± 200</td>
<td>1500 ± 300</td>
<td>2000 ± 400</td>
<td>2500 ± 500</td>
</tr>
<tr>
<td>Phase II τ, s</td>
<td>22 ± 2</td>
<td>17 ± 1</td>
<td>12 ± 1</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>Primary amplitude</td>
<td>2716 ± 398</td>
<td>2636 ± 486</td>
<td>2450 ± 497</td>
<td>2264 ± 386</td>
</tr>
<tr>
<td>Slow component</td>
<td>886 ± 235</td>
<td>881 ± 259</td>
<td>302 ± 290</td>
<td>301 ± 274</td>
</tr>
<tr>
<td>Amplitude</td>
<td>398 ± 8</td>
<td>398 ± 8</td>
<td>62 ± 12</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>End exercise</td>
<td>179 ± 12</td>
<td>1205 ± 25</td>
<td>145 ± 30</td>
<td>158 ± 16</td>
</tr>
<tr>
<td>Phase II τ, s</td>
<td>30 ± 6</td>
<td>28 ± 9</td>
<td>35 ± 14</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>Primary amplitude</td>
<td>2716 ± 398</td>
<td>2636 ± 486</td>
<td>2450 ± 497</td>
<td>2264 ± 386</td>
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<tr>
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</tr>
<tr>
<td>Amplitude</td>
<td>398 ± 8</td>
<td>398 ± 8</td>
<td>62 ± 12</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. #P < 0.05 compared to H-PL. *P < 0.05 compared to N-BR. †P < 0.05 compared to N-PL.
likely reflective of the dynamic balance between NOS-derived NO and NO$_2$ reduction to NO. In the present study, plasma [NO$_2$] declined during both moderate- and severe-intensity exercise (Fig. 1) with the magnitude and rate of plasma [NO$_2$] decline being significantly greater in the BR trials compared with PL trials, in both normoxia and hypoxia. These findings suggest that the reduction of NO$_2$ to NO outweighed the synthesis of NO through NOS exercise.

The rate of plasma [NO$_2$] decline over the 5-min moderate-intensity bout was not significantly different between N-BR and H-BR, and N-PL and H-PL. However, following 5 min of moderate-intensity exercise, plasma [NO$_2$] had fallen significantly below ModBL in N-BR; whereas, there was only a trend for a lower plasma [NO$_2$] in H-BR. Similarly, the rate of plasma [NO$_2$] decline over the severe-intensity exercise bout was not significantly different between N-BR and H-BR or N-PL and H-PL, but the absolute fall in plasma [NO$_2$] tended to be less in H-BR than in N-BR, in spite of a longer exercise duration in N-BR. These results are contrary to our hypothesis and suggest that, in hypoxia, the contribution of NOS to NO production (30), and subsequently to the regulation of muscle perfusion and matching of O$_2$ supply, may be slightly greater (12).

During the 10-min passive recovery from exhaustive exercise, plasma [NO$_2$] increased in a similar fashion in H-PL and N-PL. Specifically, plasma [NO$_2$] increased after 3 min of recovery and plateaued after 10 min. The increases in plasma [NO$_2$] may represent an increase in NO oxidation (as NO is continuing to contribute to muscle perfusion and matching of O$_2$ supply and demand; Ref. 12) during recovery. Following BR supplementation, the recovery profile of plasma [NO$_2$] was slightly different between normoxia and hypoxia. Plasma [NO$_2$] was higher in H-BR than N-BR following 1.5 min of recovery, although the difference between Exh and 1.5Rec was not different between conditions. It is important to note that differences in plasma [NO$_2$] dynamics between hypoxia and normoxia were not substantial either during exercise or in recovery.

### Effects of BR Supplementation on the Physiological Response to Moderate-Intensity Exercise

BR supplementation significantly reduced the O$_2$ cost of submaximal cycle exercise in hypoxia. V˙O$_2$ during baseline cycling in H-BR was reduced by 10% compared with H-PL and by 4% compared with N-PL. Furthermore, a 7% reduction in the end-exercise (steady-state) V˙O$_2$ was found in H-BR compared with H-PL. These findings are consistent with previous studies that have reported reductions in submaximal cycling V˙O$_2$ in varying severities of hypoxia. For example, Masschelein et al. (50) reported a 4% reduction in steady-state V˙O$_2$ with an FIO$_2$ of 0.11 during cycle exercise at 45% peak V˙O$_2$, and Mugggeridge et al. (51) reported a $\sim$6–8% reduction in steady-state V˙O$_2$ at an FIO$_2$ of 0.15 during cycle exercise at 60% of maximum work rate, following BR supplementation. A reduction in muscle metabolic perturbation [i.e., slower rates of change of muscle pH and phosphocreatine (PCr) and inorganic phosphate concentrations] during severe-intensity knee-extensor exercise in hypoxia has also been reported following BR supplementation (64).

In the present study, the V˙O$_2$ phase II $\tau$ during moderate-intensity exercise was reduced by BR supplementation in hypoxia. This finding is consistent with a recent study in older individuals, where the V˙O$_2$ mean response time was speeded with BR supplementation (32). This may be related to the slower VO$_2$ kinetics that is typically found in older individuals and the potential to abate this through enhancing muscle O$_2$ delivery (57), via increasing NO bioavailability. Similarly, hypoxia tended to slow VO$_2$ kinetics in the young healthy participants in the present study. Specifically, the phase II $\tau$ tended to be slowed in hypoxia compared with normoxia (from $\sim$22 to $\sim$31 s; Table 2). This observation is consistent with previous reports of slower VO$_2$ kinetics in hypoxia (29, 59). BR supplementation speeded the phase II $\tau$ in hypoxia toward values recorded in normoxia, thereby helping to reverse the detrimental effect of a reduced FIO$_2$ on VO$_2$ kinetics. These findings are consistent with a recent study that showed muscle PCr recovery kinetics, which reflect the maximal rate of mitochondrial ATP resynthesis and are influenced by O$_2$ availability, were speeded by BR supplementation in hypoxia (64).
These data suggest that, in addition to reducing O$_2$ demand during exercise (50, 51, and present study), BR may enhance skeletal muscle O$_2$ availability in hypoxia. In contrast to some (3, 4, 14, 40, 41, 62), but not all (5, 8, 32, 66), previous studies, 3 days of BR supplementation did not significantly reduce VO$_2$ during submaximal exercise in normoxia. Previous studies have typically reported reductions in steady state VO$_2$ of 3–5% following several days of NO$_3^-$ supplementation (4, 40, 62). The mechanistic bases for this lower O$_2$ cost of exercise have been suggested to include improved mitochondrial efficiency (39) and/or reductions in the ATP cost of muscle force production (3), which may be linked to enhanced Ca$^{2+}$-related muscle contractility (28). NO is involved in the regulation of mitochondrial O$_2$ consumption, and it is well established that NO has a strong affinity for cytochrome-c oxidase (COX) (9). It has been suggested that competition for the COX binding site between NO and O$_2$ may be responsible, in part, for the reduced O$_2$ cost of exercise following NO$_3^-$ supplementation (4, 41), with this initiating a signaling cascade resulting in mitochondrial protein changes, which collectively enhance respiratory chain efficiency (39). Interestingly, hypoxia, per se, may also result in an acute, reversible inhibition of COX (10). The combination of hypoxia and BR supplementation may, therefore, make it more likely for these effects to be manifest. It is also noteworthy that reductions in VO$_2$ during moderate-intensity exercise were recently reported to be evident following acute supplementation with 16.8 mmol NO$_3^-$ (4 x 70 ml BR shots), tended to be evident with 8.4 mmol NO$_3^-$ (2 x 70 ml BR shots), but were not evident with 4.2 mmol NO$_3^-$ (1 x 70 ml BR shot) (67). It is, therefore, possible that an insufficient NO$_3^-$ dose was consumed immediately prior to the tests to significantly influence the VO$_2$ response to exercise in normoxia in the present study. Furthermore, the interindividual differences in the VO$_2$ response to exercise in normoxia evident in the current study, may have also contributed to the lack of statistically significant effects. It may be concluded that BR supplementation can (3, 4, 14, 40, 41, 62), but does not always (present study, 5, 8, 32, 66), alter the O$_2$ cost of exercise in normoxia.

Indices of muscle oxygenation measured with NIRS were altered as a result of the manipulation of Fio$_2$ during moderate-intensity exercise but BR supplementation did not significantly influence this response. Consistent with a previous study (50), [HHb] was greater in hypoxia indicating that muscle fractional O$_2$ extraction was increased, while [HbO$_2$] and TOI were significantly reduced in hypoxia compared with normoxia. Although not significant, BR supplementation tended to ameliorate the negative effects of hypoxia upon TOI during moderate-intensity exercise in the current study (a 3.6% increase in TOI), in a similar fashion to that reported by Masschelein et al. (50) (a 4% increase in TOI). These effects are consistent with observations that the arterial-venous [NO$_2^-$] difference is associated with limb vasodilatation and increased skeletal muscle blood flow during exercise performed in hypoxia (20). The trend for an improved TOI with BR supplementation indicates better muscle oxygenation (24), which may have been responsible for the speeding of the VO$_2$ kinetics observed in hypoxia. Consistent with a possible improvement in oxygenation status, the typical compensatory rise in HR in hypoxia was attenuated by BR compared with PL during moderate-intensity exercise. Specifically, HR was 5–6 beats/min lower in the H-BR compared with the H-PL condition. There were no differences between H-BR and H-PL in indices of muscle oxygenation or HR during severe-intensity exercise.

Whether the reduction in cardiac work (lower HR) and metabolic requirement (lower VO$_2$) with BR observed in the present study might translate into enhanced performance during prolonged low-intensity exercise at altitude remains to be determined. Furthermore, older age and a number of disease conditions, including peripheral arterial disease, diabetes, COPD, and anemia, are associated with tissue hypoxia. A reduced O$_2$ cost of moderate-intensity exercise (i.e., walking) and reduced muscle metabolic perturbation during physical activity may improve the quality of life in individuals with these diseases (34, 64). However, further research is required to explore the effects of BR supplementation on health and functional capacity in patient populations.

Effects of BR Supplementation on the Physiological Response to Severe-Intensity Exercise

The end-exercise VO$_2$ was significantly reduced during severe-intensity exercise in hypoxia compared with normoxia. Moreover, [HbO$_2$] and TOI of the m. vastus lateralis were significantly reduced, while [HHb] and HR were significantly increased in hypoxia compared with normoxia, consistent with previous findings (50). There was a trend toward a reduction in end-exercise VO$_2$ with BR compared with PL supplementation in hypoxia of ~6%. This finding indicates the VO$_2$ peak may be reduced by NO$_3^-$ supplementation and is consistent with some (6, 42) but not all previous studies (4, 33, 62) conducted in normoxia.

Tolerance to severe-intensity cycle exercise in hypoxia in the present study was significantly improved (9%, P < 0.05) following BR supplementation. This finding is consistent with earlier studies that reported that BR supplementation increased exercise tolerance during constant-work-rate (64) and incremental (50) exercise protocols and enhanced cycling time-trial performance (51) in hypoxia. However, in contrast to previous findings (3, 4, 8, 33, 37), we found no effect of BR supplementation on exercise tolerance in normoxia. An interesting observation in the present study was the significant correlation between the reduction in steady-state VO$_2$ and the improvement in exercise tolerance following BR supplementation in hypoxia (r = −0.96). Therefore, the lack of effect on VO$_2$ during submaximal exercise in normoxia following BR supplementation may explain the lack of effect on exercise tolerance. Further research is required to address the physiological bases for responders and nonresponders to dietary nitrate supplementation.

Perspectives and Significance

This study provides the first description of the influence of Fio$_2$ and BR supplementation on plasma [NO$_2^-$] dynamics during moderate- and severe-intensity exercise and subsequent recovery in humans. The greater rate of decline of plasma [NO$_2^-$] during exercise following BR compared with PL supplementation suggests that elevating plasma [NO$_2^-$] prior to exercise may promote NO production through the nitrate-nitrite-NO pathway. In hypoxia, but not normoxia, BR supplementation reduced the O$_2$ cost of moderate-intensity exercise, speeded VO$_2$ kinetics, and improved severe-intensity exercise.
tolerance. These findings may have important implications for individuals exercising at altitude.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.K., J.V., S.J.B., L.J.W., P.G.W., and A.M.J. conceived and designed the research; J.K., L.J.W., C.T., and S.L. performed experiments; J.K., C.T., used in this study, gratis.

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