Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia

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Masschelein E, Van Thienen R, Wang X, Van Schepdael A, Thomis M, Hespel P. Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. J Appl Physiol 113: 736–745, 2012. First published July 5, 2012; doi:10.1152/japplphysiol.01253.2011.—Exercise tolerance is impaired in hypoxia, and it has recently been shown that dietary nitrate supplementation can reduce the oxygen (O2) cost of muscle contractions. Therefore, we investigated the effect of dietary nitrate supplementation on arterial, muscle, and cerebral oxygenation status, symptoms of acute mountain sickness (AMS), and exercise tolerance at simulated 5,000 m altitude. Fifteen young, healthy volunteers participated in three experimental sessions according to a crossover study design. From 6 days prior to each session, subjects received either beetroot (BR) juice delivering 0.07 mmol nitrate/kg body wt/day or a control drink (CON). One session was in normoxia (NORCON); the two other sessions were in hypoxia (11% O2), with either CON (HYPCON) or BR (HYPBR). Subjects first cycled for 20 min at 45% of peak O2 consumption (VO2peak; EX45%) and thereafter, performed a maximal incremental exercise test (EXmax). Whole-body VO2, arterial O2 saturation (%SpO2) via pulsoximetry, and tissue oxygenation index of both muscle (TOIM) and cerebral (TOIC) tissue by near-infrared spectroscopy were measured. Hypoxia per se substantially reduced VO2peak, %SpO2, TOIsa, and TOIC (NORCON vs. HYPCON, P < 0.05), whereas %SpO2 was higher (P < 0.05), whereas %SpO2 was higher (P < 0.05), whereas %SpO2 was higher (P < 0.05), TOIsa was ~5% higher in HYPBR than in HYPCON both at rest and during EX45% and EXmax (P < 0.05), TOIC as well as the incidence of AMS symptoms were similar between HYPCON and HYPBR at any time. Hypoxia reduced time to exhaustion in EXmax by 36% (P < 0.05), but this ergolytic effect was partly negated by BR (+5%, P < 0.05).

Short-term dietary nitrate supplementation improves arterial and muscle oxygenation status, but not cerebral oxygenation status during exercise in hypoxia. This is associated with improved exercise tolerance against the background of a similar incidence of AMS.

NITRIC OXIDE (NO) IS IMPLICATED in a multiplicity of physiological processes in the human body. Amongst others, this includes vascular control, regulation of muscle contractility, mitochondrial respiration, glucose and calcium homeostasis, and hypoxic vasodilation (14, 15, 23, 39, 47). NO is endogenously produced by the action of the NO synthase enzyme (NOS) on circulating l-arginine. Alternatively, NO can also be produced from dietary nitrate. Nitrate is a natural ingredient in vegetables and is particularly abundant in leafy greens and beetroots (27). A fraction of the nitrate ingested is reduced to nitrite by facultative anaerobic bacteria in the saliva and further converted to NO in the acid environment of the stomach (7).

Interestingly, recent data have provided substantial evidence to indicate that dietary nitrate supplementation in young, healthy volunteers, either in the form of pure sodium nitrate (35, 36) or beetroot (BR) juice (2, 3, 33, 50), reduces the oxygen (O2) cost of a given intensity of muscle contractions. This translates into higher fraction of oxygenated hemoglobin in muscle (3), as well as lower rate of whole-body O2 uptake (VO2) in endurance exercise (2, 3, 33, 35, 36, 50). The exact physiological mechanisms underlying this “ergogenic” action are only partly understood. However, the observation that l-arginine supplementation, which also may increase NO production but via another route other than nitrate intake, in one study (4) yielded similar ergogenic effects as nitrate intake also supports the assumption that NO is probably the key molecule involved. NO may impact on mitochondrial function via different mechanisms (15, 34), but also, nitrite has the potential to regulate tissue protein expression and activity directly (11). Accordingly, a recent study has shown that nitrate administration in healthy volunteers upregulates mitochondrial oxidative phosphorylation efficiency, which probably is at least partly explained by reduced proton leakage across the inner mitochondrial membrane due to downregulation of the ATP/ADP translocase protein. Furthermore, it has also been shown that dietary nitrate supplementation reduces the ATP cost of muscle contractions (2), which might be linked to a reduced ATP expenditure on maintenance of sarcoplasmic calcium homeostasis (47). Furthermore, dietary nitrate supplementation also improved high-intensity exercise tolerance, which could be due to enhanced oxidative energy turnover in conjunction with better muscle perfusion due to NO-induced vasodilation during the transition from rest to exercise (14, 23).

Recent observations also indicate that NO is an important signaling molecule in adaptation to altitude/hypoxia (37). For instance, altitude residents (~Tibetans) exhale higher concentrations of NO (6) and have many-fold higher plasma nitrite levels than lowlanders (20). Furthermore, when lowlanders are exposed to acute hypoxia, the fraction of NO in the expired air decreases (10, 19), which may be a maladaptive response to hypoxia. In addition, a number of studies have demonstrated that lower exhaled NO levels are associated with a higher incidence of altitude illnesses (16, 18). A recent study also showed that dietary nitrate reduces muscle metabolic perturbation during exercise in hypoxia (51). Given the evidence for a beneficial role of NO in hypoxia, it is reasonable to hypothesize that dietary nitrate supplementation could be more potent

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to enhance exercise performance in hypoxia than in normoxia. An additional argument to support such hypothesis is the fact that NO formation via the l-arginine NOS pathway is inhibited in hypoxic conditions (40). In such condition, the nitrate–nitrite–NO pathway probably could serve as a back-up system to maintain sufficient NO levels when O2 supply is limited.

Against this background, the aim of the present study was to investigate the effect of short-term dietary nitrate supplementation in the form of BR juice on muscle oxygenation status and exercise capacity at high altitude (5,000 m, 11% O2). Because exercise performance at high altitude is often limited even more by acute mountain sickness (AMS) than by muscular deficits per se, we also evaluated the effect of nitrate administration on cerebral oxygenation status and symptoms of AMS. In this regard, we postulated that the reduced O2 cost of submaximal exercise at high altitude could facilitate maintenance of higher arterial partial pressure of O2 (pO2) and thereby inhibit the development of AMS.

MATERIALS AND METHODS

Subjects. Fifteen healthy, physically active males [age: 21.1 ± 1.0 yr, body wt: 72.3 ± 2.5 kg; peak VO2 (VO2peak): 61.7 ± 2.1 ml kg–1 min–1] volunteered to participate in the study, which was approved by the KU Leuven Biomedical Ethics Committee and was in accordance with The Declaration of Helsinki. Subjects gave their written, informed consent after they were fully informed of all experimental procedures and risks associated with the experiments. None of the subjects were tobacco smokers, used dietary supplements, or were exposed to an altitude higher than 1,500 m within 6 mo prior to the study. All subjects were asked to continue their normal sports and other physical activities throughout the study, and they were instructed to refrain from heavy exercise and alcohol for 24 h prior to the experimental sessions. Furthermore, the subjects received a list of nitrate-rich foods to be avoided during the entire study period so as to reduce the amount of involuntary dietary nitrate intake. On the evening before the experiments, the subjects received a standardized dinner (1,000 kcal, carbohydrates 60 percent total energy content (60%E)–fat 27%E–protein 13%E), and the next morning, they consumed a standardized breakfast (700 kcal, carbohydrates 85%E–fat 9E%–protein 6E%), ~3 h before the start of the exercise tests, from which time, the use of caffeine-containing nutrients was also prohibited. All tests were performed in the Research Center for Exercise and Health, KU Leuven.

Study protocol. The subjects participated in three experimental exercise sessions in a normobaric hypoxic facility (SportingEdge, Sherfield on Loddon, UK), according to a single blind, randomized crossover study design, and with a 2-wk washout period between the experimental sessions. Prior to each experimental session, the subjects underwent a 6-day dietary supplementation with either BR juice containing 0.07 mmol nitrate/kg body wt/day or an equivalent volume (~500 ml) of apple-blackcurrant juice containing ~5 μmol nitrate/kg body wt/day, which served as a control drink (CON). Energy (kcal) content in the form of carbohydrates, fats, and proteins was rather similar between BR and CON. One CON session was performed in normoxia (NORMCON: 20.93% ambient O2); the two other sessions were done in severe hypoxia (11% ambient O2, corresponding to ~5,000 m altitude)—one session with CON (HYPCon) and the other with BR (HYPBR). The subjects were not aware of the experimental hypotheses and were disinfomed on the true composition of the juices. Subjects were instructed to distribute the intake over five equal doses (~100 ml) throughout the day. However, on the day of the experiments, they received the full dose (~500 ml) between 1 and 2 h before the start of the experimental session. During the experimental sessions, the O2 content in the hypoxic facility was automatically controlled at the pre-set level (20.93% or 11%), and ambient air temperature and relative humidity were maintained at 20°C and 50%, respectively. Two weeks before the start of the study, all subjects performed a familiarization session in the hypoxic facility, set at normoxia, to become familiar with all experimental procedures. At this occasion, a maximal incremental exercise test (EXmax) was also performed on a cycle ergometer (Avantronic Cyclus II, Leipzig, Germany) to determine VO2peak and peak workload, as well as to fix the bike position to be reproduced in the next experimental sessions.

Experimental sessions. For each experimental session, the subjects reported to the laboratory in the morning between 8 and 9 AM. Upon arrival, they rested for 1 h in a comfortable chair, where after resting, blood pressure was measured with a cuff placed on the upper right arm using an electronic sphygmomanometer (Omrón M6, Omron Healthcare, Kyoto, Japan). Blood pressure was measured four times at 1-min intervals, and a mean value was calculated using only the last three values measured. Basal pulmonary gas-exchange parameters were then measured during a 5-min period using an open circuit ergospirometry device (Cortex MetaLyzer II, Leipzig, Germany). Finally, a capillary blood sample was collected from the ear lobe for determination of blood lactate, and a blood sample (10 ml) was taken from an arm vein into lithium-heparin tubes for later analysis of plasma nitrate and nitrite. Following these baseline measurements, a cycling exercise protocol (Avantronic Cyclus II) was started. Subjects successively performed a 20-min submaximal constant-load cycling bout at a workload corresponding to 45% of their previously determined VO2peak (EX45%) and an EXmax test to volitional exhaustion (50 W + 20 W/min), with a 15-min rest interval in between. From the start to the end of the experimental session, heart rate (HR) was measured continuously (Polar, Kempele, Finland), and arterial O2 saturation (%SpO2) was monitored using a pulsoximeter (Nellcor N-600-x, Oxismart, Mallinckrodt, St. Louis, MO) with a sensor placed on the forehead, ~2 cm above the right eyebrow. Local oxygenation status in the right m. vastus lateralis and left prefrontal cortex was measured continuously by means of near-infrared spectroscopy (NIRS: NIR-O200, Hamamatsu, Japan; see below for further details).

During all exercise tests, pulmonary gas-exchange parameters were measured continuously, and capillary blood samples for lactate assay were collected each 5 min in EX45% and 2 min after the point of exhaustion in EXmax. A 15-point Borg scale (6–20) was also used to measure ratings of perceived exertion (RPE) immediately after EX45% and EXmax. Following EXmax, the subjects recovered for 30 min in a comfortable chair, after which AMS, symptoms were assessed using the Lake Louise Questionnaire. Presence and severity of symptoms inherent to AMS-like headache, gastrointestinal symptoms, fatigue, and dizziness were evaluated. Each symptom was graded on a scale ranging from zero (absent) to three (most severe). According to the Lake Louise AMS score guidelines, AMS is present when subjects suffer from headache and at least one other symptom, with a total score of three or more (45).

NIRS. NIRS has become a popular method to measure tissue oxygenation status noninvasively (8). The principles, limitations, and reliability of NIRS measurements have been described extensively elsewhere (22). We used the NIRO-200 NIRS instrument (Hamamatsu) for the purpose of this study. One pair of NIRS probes, consisting of one light emitter and one light detector, was attached on the belly of the right m. vastus lateralis in parallel with the long axis of the muscle. The other pair of NIRS probes was attached on the left prefrontal cortex, ~2 cm above the left eyebrow as laterally as possible. The probes were fitted in a dark-colored plastic spacer with a fixed interoptode distance of 4 cm and attached to the skin using double-sided adhesive tape. Elastic, noncompressive bandages were used to keep the probes in place. Probe placement was made in an upright standing position before the experimental session started. Before positioning of the probes, the skin was shaved to exclude interaction of hair as a chromophore. The contour lines of the two probes were drawn on the skin with a permanent marker to allow identical
repositioning of the probes during each subsequent session. NIRO-200 applies three different wavelengths of near-infrared light and provides real-time information on the dynamic balance between O₂ supply and VO₂. The so-called “tissue oxygenation index” (TOI), as determined by spatially resolved spectroscopy, is a valid parameter (8, 42) to assess the fraction of O₂-saturated tissue hemoglobin and myoglobin, reflecting tissue oxygenation status. Furthermore, changes in the fractions of oxygentated (Δ[O₂Hb]) and deoxygenated (Δ[Hb]) hemoglobin relative to baseline were estimated using the Beer-Lambert Law. Changes in the fractions of total hemoglobin (Δ[THb]) were calculated as the sum of Δ[O₂Hb] and Δ[Hb] and used as an index of change in regional blood volume (49). We decided not to use a differential path-length factor (DPF) for either muscle or cerebral measurements because DPF may vary from one wavelength to another, and across subjects and tissue (17), thereby biasing depth of the NIRS signal is approximately one-half of the interoptode distance (4.0 cm) and because adipose tissue thickness on the other, and across subjects and tissue (17), Δ[O₂Hb] and Δ[Hb] data are expressed in micromoles-centimeters (μM·cm). Since the sampling depth of the NIRS signals is approximately one-half of the interoptode distance (4.0 cm) and because adipose tissue thickness over the m. vastus lateralis, on average, was only 3.9 mm (range: 2.4–5.5), it can be assumed that the captured NIRS signals largely reflected the hemodynamic status of the muscle tissue underlying the probes (22). The NIRS data were recorded continuously with a sampling frequency of 2 Hz and were preprocessed with a Butterworth filter in customer-level mathematical software, and average values/5-s window were calculated (Matlab, MathWorks, Natick, MA). Δ[O₂Hb], Δ[Hb], and Δ[THb] (μM·cm) values were expressed relative to the baseline value measured during the final 10 min of the post-exercise rest period.

Blood and plasma analysis. Capillary blood samples from the ear lobe were analyzed immediately for blood lactate concentration using an automated lactate analyzer (Lactate Pro, Arkray, Japan). Venous blood samples were centrifuged immediately (4,000 rpm for 10 min at 4°C), and plasma was separated to be stored at −80°C for later analysis of nitrate and nitrite. The determination of nitrate and nitrite was carried out by capillary zone electrophoresis (CZE) on HP3DCE equipment (Agilent, Waldbronn, Germany), as described in detail elsewhere (54). Briefly, field-amplified sample stacking was used to achieve submicromolar detection by dilution of the plasma sample with deionized water. In CZE, the separation of nitrate and nitrite was achieved within 10 min without adding an electro-osmotic flow modifier. The optimal condition was achieved with 50 mM phosphate buffer at pH 9.3 as a background electrolyte. The separation voltage was 20 kV (negative potential), and UV detection was performed at 214 nm. The temperature of the cassette was kept at 40°C. After thawing at room temperature, 150 μl plasma sample was deproteinized by spiking with 5 μl 1 M NaOH to pH 10 and then centrifuged at 14,100 g for 10 min in a MiniSpin Plus microcentrifuge (Eppendorf, Hamburg, Germany). Then, the ninefold-diluted plasma samples (100 μl supernatant with 800 μl water) were injected hydrodynamically for 40 s into a 60-μm × 75-μm internal diameter uncoated fused-silica capillary. The capillary was flushed between runs with 0.1 M NaOH (2 min), distilled water (2 min), and running buffer (2 min). Thiocyanate was used as internal standard at a concentration of 10 μM. The linearity curves were obtained with the standard addition method, and the estimated limits of detection were 0.07 and 0.05 μM in ninefold-diluted plasma sample vs. 0.63 and 0.45 μM in original plasma for nitrate and nitrite, respectively. The intra- and interday precision for both analytes was <2.6%, and the recovery ranged between 92% and 113%. Measured values are consistent with literature data (24).

Data analysis. Before statistical analysis and to facilitate interpretation of the data, mean values for HR, gas-exchange parameters, %SpO₂, and the NIRS values were calculated for: 1) the last 10 min of the rest period, 2) the last 10 min of EX₄₅%, and 3) the final 30 s of EX₄₅%. These values represent the status during rest, submaximal exercise, and maximal exercise, respectively. Furthermore, energy expenditure during the last 10 min of EX₄₅% was calculated from VO₂ and carbon dioxide production (VCO₂) using the formula of Brouwer (9): energy expenditure (Jls) = [(3.869 × VO₂) + (1.195 × VCO₂)] × (4.186/60) × 1,000; and net mechanical efficiency (NE) was calculated: NE (%) = work rate (W)/[energy expenditure (Jls) − energy expenditure at rest (Jls)] × 100.

Statistics. Separate analyses were run to evaluate the earlier stated hypotheses: 1) the effects of nitrate per se (NORCON vs. HYPCON) and 2) to compare the effects of BR vs. CON in hypoxia (HYPCON vs. HYPBR). Two-tailed paired t-tests were used for HR, exercise capacity, resting and peak blood lactate, gas-exchange parameters, NE, blood pressure, plasma nitrate and nitrite, RPE, and AMS to compare NORCON vs. HYPCON on the one hand and HYPCON vs. HYPBR on the other hand. Exercise-induced changes in NIRS variables, %SpO₂, and blood lactate during EX₄₅% were evaluated by a two-way repeated measures ANOVA to determine the main effects of conditions (F₁: NORCON vs. HYPCON, HYPCON vs. HYPBR) as well as to assess the condition × time interaction (F₁). As a post hoc comparison, a Bonferroni t-test was used when appropriate. A Bonferroni t-test was also used to evaluate specific preplanned comparisons. A probability level P < 0.05 was considered statistically significant. All data are expressed as means ± SE.

RESULTS

Plasma nitrate and nitrite. Plasma nitrate and nitrite were similar between NORCON and HYPCON (Table 1). Compared with HYPCON, in HYPBR, plasma nitrate was increased ~3.5-fold (P < 0.05), whereas plasma nitrite was increased by ~40% (P < 0.05).

HR, blood lactate, and exercise capacity. Compared with NORCON, in HYPCON, HR at rest and during EX₄₅% was increased substantially, whereas peak HR (HRpeak) was reduced (Table 2). HR was similar between HYPCON and HYPBR at all times. Compared with NORCON (1.3 ± 0.1 mmol/l), blood lactate at rest was higher in both HYPCON (1.9 ± 0.1 mmol/l, P < 0.05) and HYPBR (1.7 ± 0.1 mmol/l, P < 0.05). Blood lactate in NORCON did not increase during EX₄₅% (Fig. 1). However, in HYPCON, lactate increased progressively to ~5–6 mmol/l. Throughout EX₄₅%, blood lactate was lower in HYPBR than in HYPCON (F₁ = 4.8, P < 0.05), yet the difference was only significant from 10 to 15 min (P < 0.05). During EX₄₅%, compared with NORCON (888 ± 37 s), time to exhaustion in HYPCON (568 ± 23 s) was decreased by 36% (P < 0.05). In HYPBR (597 ± 22 s), time to exhaustion was slightly higher (+5%, P < 0.05; Fig. 2) than in HYPCON. In fact, in 13 out of 15 subjects, time to exhaustion was longer in HYPBR than in HYPCON (Fig. 2). Blood lactate during EX₄₅% peaked at 10.6 ± 0.3 mmol/l in NORCON vs. 9.1 ± 0.5 mmol/l in HYPCON (P < 0.05). Peak lactate was similar between HYPCON and HYPBR (9.6 ± 0.5 mmol/l).

Table 1. Effect of nitrate supplementation on plasma nitrate and nitrite

<table>
<thead>
<tr>
<th></th>
<th>NORCON</th>
<th>HYPCON</th>
<th>HYPBR</th>
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<tbody>
<tr>
<td>Nitrate (μmol/l)</td>
<td>40 ± 3</td>
<td>41 ± 3</td>
<td>147 ± 7*</td>
</tr>
<tr>
<td>Nitrite (μmol/l)</td>
<td>3.7 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>5.3 ± 0.3*</td>
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</tbody>
</table>

Values are means ± SE (n = 10) in normoxia with a control drink (NORCON), hypoxia with CON (HYPCON), and hypoxia with beetroot juice delivering 0.07 mmol nitrate/kg body w/day (HYPBR). Plasma nitrate and nitrite were measured in NOR and in 11% ambient oxygen (O₂) content (HYP) in the presence (HYPBR) or absence (NORCON and HYPCON) of dietary nitrate supplementation. *P < 0.05 compared with NORCON and HYPCON.
Table 2. Effect of nitrate supplementation in hypoxia on pulmonary gas-exchange parameters at rest and during exercise

<table>
<thead>
<tr>
<th></th>
<th>NORCON</th>
<th>HYPCON</th>
<th>HYPBR</th>
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<tbody>
<tr>
<td><strong>REST</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VE (l/min)</td>
<td>8.6 ± 0.5</td>
<td>11.8 ± 0.5</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>VO2 (l/min)</td>
<td>0.39 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>VCO2 (l/min)</td>
<td>0.32 ± 0.03</td>
<td>0.39 ± 0.01</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>RER</td>
<td>0.84 ± 0.03</td>
<td>0.90 ± 0.02*</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>64 ± 3</td>
<td>78 ± 3*</td>
<td>75 ± 3</td>
</tr>
<tr>
<td><strong>EX45%</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VE (l/min)</td>
<td>40.6 ± 1.5</td>
<td>61.7 ± 2.0</td>
<td>59.4 ± 1.5</td>
</tr>
<tr>
<td>VO2 (l/min)</td>
<td>2.09 ± 0.07</td>
<td>2.03 ± 0.06</td>
<td>1.95 ± 0.06</td>
</tr>
<tr>
<td>VCO2 (l/min)</td>
<td>1.86 ± 0.07</td>
<td>1.97 ± 0.06*</td>
<td>1.89 ± 0.05</td>
</tr>
<tr>
<td>RER</td>
<td>0.89 ± 0.01</td>
<td>0.97 ± 0.02*</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>124 ± 3</td>
<td>150 ± 2*</td>
<td>148 ± 3</td>
</tr>
<tr>
<td><strong>EXmax</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VEpeak (l/min)</td>
<td>130 ± 7.3</td>
<td>121.0 ± 7.2*</td>
<td>118.9 ± 5.9</td>
</tr>
<tr>
<td>VO2peak (l/min)</td>
<td>4.44 ± 0.15</td>
<td>2.88 ± 0.14*</td>
<td>2.79 ± 0.11</td>
</tr>
<tr>
<td>VCO2peak (l/min)</td>
<td>4.90 ± 0.17</td>
<td>3.30 ± 0.12*</td>
<td>3.27 ± 0.12</td>
</tr>
<tr>
<td>RERpeak</td>
<td>1.11 ± 0.02</td>
<td>1.16 ± 0.04</td>
<td>1.18 ± 0.03</td>
</tr>
<tr>
<td>HRpeak (beats/min)</td>
<td>191 ± 3</td>
<td>173 ± 2*</td>
<td>173 ± 3</td>
</tr>
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</table>

Values are means ± SE (n = 15) for minute ventilation (VE), O2 uptake (VO2), carbon dioxide output (VCO2), respiratory exchange ratio (RER), and heart rate (HR) during a constant-load exercise bout at 45% of peak VO2 (VO2peak; EX45%) and at peak workload during an incremental exercise test (EXmax). Subjects performed 1 experimental session in NOR and 1 session in 11% ambient O2 content (HYP) in the presence (HYPBR) or absence (NORCON and HYPCON) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with NORCON. †P < 0.05 compared with HYPBR.

Pulmonary gas exchange and NE. Compared with NORCON, in HYPCON at rest and during EX45%, ventilation (VE) (P < 0.05), VO2 (P < 0.05), and respiratory exchange ratio (RER; P < 0.05) were increased, whereas VO2 was unchanged (Table 2). VO2 was lower in HYPBR than in HYPCON, both at rest (−8%, P < 0.05) and during EX45% (−4%, P < 0.05). VCO2 at rest was similar between HYPCON and HYPBR, but during EX45%, VCO2 was slightly lower (−5%, P < 0.05) in the latter. RER and VE were similar between HYPCON and HYPBR. During EXmax and compared with NORCON, VO2peak (−35%, P < 0.05) peak VCO2 (VCO2peak; −33%, P < 0.05), and peak VE (VEpeak; −7%, P < 0.05) were reduced in HYPCON, yet peak RER (RERpeak) was similar. VO2peak, VCO2peak, RERpeak, and VEpeak were similar between HYPCON and HYPBR. Furthermore, no difference was found in NE between NORCON (18.6 ± 0.5%) and HYPCON (19.0 ± 0.5%). However, NE tended to be higher in HYPBR (19.9 ± 0.5%, P = 0.07) than in HYPCON.

Muscle oxygenation status. In normoxia (NORCON), %SO2 was mostly stable, although slightly dropping at EXmax. Conversely, in hypoxia (HYPCON and HYPBR), %SO2 started from a substantially lower baseline level and dropped further during exercise. From these curves, mean values (see MATERIALS AND METHODS for details) were calculated for rest, EX45%, and EXmax (Fig. 3B). Compared with NORCON, %SO2 in HYPCON was lower at rest (−22%, P < 0.05) and during both EX45% (−32%, P < 0.05) and EXmax (−27%, P < 0.05). Compared with HYPCON, %SO2 was slightly increased in HYPBR both at rest (+3.5%, P < 0.05) and during EX45% (+2.7%, P < 0.05) but not during EXmax.

Fig. 1. Effect of nitrate supplementation in hypoxia on blood lactate during constant-load exercise. Values are means ± SE (n = 15) in normoxia with a control drink [NORCON (○)], hypoxia with CON [HYPCON (■)], and hypoxia with beetroot (BR) juice delivering 0.07 mmol nitrate/kg body wt/day [HYPBR (×)]. Subjects performed a 20-min submaximal constant-load exercise bout at 45% of peak oxygen (O2) consumption (EX45%) in normoxia (○) and in 11% ambient O2 content (■, ×) in the presence (○, ■) or absence (×, ×) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with NORCON; †P < 0.05 compared with HYPBR.

Fig. 2. Effect of nitrate supplementation in hypoxia on exercise capacity. Individual data points (dotted lines) and mean ± SE (n = 15; solid line) are given. Time to exhaustion was measured during a maximal incremental exercise test (EXmax) to volitional exhaustion in 11% ambient O2 content in the presence (HYPBR) or absence (HYPCON) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with HYPCON.

Muscle oxygenation status. In normoxia (NORCON), TOI decreased during EX45% and dropped further during EXmax. In hypoxia (HYPCON and HYPBR), compared with NORCON, TOI started from a lower baseline level, and in addition, the exercise-induced deoxygenation during EX45%, but not during EXmax, was markedly greater (Fig. 4A). From these curves, mean values (see MATERIALS AND METHODS for details) were calculated for rest, EX45%, and EXmax (Fig. 4B). Compared with NORCON, in HYPCON, TOI was lower during rest (−11%, P < 0.05), EX45% (−27%, P < 0.05), and EXmax (−18%, P < 0.05). Compared with HYPCON, muscle oxygenation status was improved in HYPBR (Fe = 14.9, P < 0.05). Thus TOIs in
HYPBR at rest (+4%, $P < 0.05$), during EX$_{45\%}$ (+4%, $P < 0.05$), as well as during EX$_{\text{max}}$ (+5%, $P < 0.05$) were higher than in HYPCON. Besides TOIs, exercise-induced $\Delta$[O$_2$Hb], $\Delta$[HHb], and $\Delta$[THb] were also evaluated (Table 3). As expected, the exercise-induced drop in [O$_2$Hb] ($F_c = 40.3$, $P < 0.05$; $F_i = 16.0$, $P < 0.05$) and the increase in [HHb] ($F_c = 16.3$, $P < 0.05$; $F_i = 20.5$, $P < 0.05$) during both EX$_{45\%}$ and EX$_{\text{max}}$ were larger in HYPCON than in NORCON. $\Delta$[O$_2$Hb] was similar between HYPCON and HYPBR at all times. However, compared with HYPCON, the exercise-induced increase in [HHb] during EX$_{45\%}$ ($P < 0.05$) and EX$_{\text{max}}$ ($P < 0.05$) was lower in HYPBR. Exercise (EX$_{45\%}$ and EX$_{\text{max}}$) increased [THb] to the same degree in all conditions.

Cerebral oxygenation status. The typical time course of cerebral TOI in the prefrontal cortex is given in Fig. 5A and Table 4. In normoxia (NORCON), TOI was constant during EX$_{45\%}$ yet decreased slightly at EX$_{\text{max}}$. Similar to muscle TOI, the exercise-induced deoxygenation in the prefrontal cortex was also greater in hypoxia (HYPCON and HYPBR) than in normoxia (NORCON). Again, from these curves, mean values (see MATERIALS AND METHODS for details) were calculated for rest, EX$_{45\%}$, and EX$_{\text{max}}$ (Fig. 5B). Compared with NORCON, TOI was lower in HYPCON at rest ($-18\%$, $P < 0.05$), during EX$_{45\%}$ ($-28\%$, $P < 0.05$), and during EX$_{\text{max}}$ ($-27\%$, $P < 0.05$). However, there were no differences between HYPCON and HYPBR at any time. Furthermore, compared with NORCON, the...
Table 3. Effect of nitrate supplementation in hypoxia on changes in oxygenated and deoxygenated hemoglobin content in m. vastus lateralis during exercise

<table>
<thead>
<tr>
<th></th>
<th>NORCON</th>
<th>HYPCON</th>
<th>HYPBR</th>
</tr>
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<tbody>
<tr>
<td>Δ[O₂Hb]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EX₄₅%</td>
<td>42 ± 21</td>
<td>−189 ± 28*</td>
<td>−162 ± 28</td>
</tr>
<tr>
<td>EXmax</td>
<td>−138 ± 23</td>
<td>−271 ± 45*</td>
<td>−222 ± 32</td>
</tr>
<tr>
<td>Δ[HHb]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EX₄₅%</td>
<td>159 ± 23</td>
<td>397 ± 51*</td>
<td>348 ± 50†</td>
</tr>
<tr>
<td>EXmax</td>
<td>391 ± 38</td>
<td>494 ± 51*</td>
<td>450 ± 42†</td>
</tr>
<tr>
<td>Δ[THb]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EX₄₅%</td>
<td>201 ± 27</td>
<td>209 ± 51</td>
<td>186 ± 33</td>
</tr>
<tr>
<td>EXmax</td>
<td>252 ± 32</td>
<td>223 ± 35</td>
<td>228 ± 22</td>
</tr>
</tbody>
</table>

Values (µM · cm) are means ± SE (n = 15) during a constant-load EX₄₅% and at EXmax. Oxyhemoglobin ([O₂Hb]), deoxyhemoglobin ([HHb]), and total hemoglobin ([THb]) content was measured by near-infrared spectroscopy (NIRS) and is expressed relative to the baseline value (Δ). Subjects performed 1 experimental session in NOR and 1 session in 11% ambient O₂ content (HYP) in the presence (HYPBR) or absence (NORCON and HYPCON) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with NORCON; †P < 0.05 compared with HYPCON.

Values (µM · cm) are means ± SE (n = 15) during a constant-load EX₄₅% and at EXmax. [O₂Hb], [HHb], and [THb] content was measured by NIRS and is expressed relative to the baseline value (Δ). Subjects performed 1 experimental session in NOR and 1 session in 11% ambient O₂ content (HYP) in the presence (HYPBR) or absence (NORCON and HYPCON) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with NORCON.

DISCUSSION

Nowadays, many individuals are regularly exposed to exercise in hypoxia, for example, during skiing or hiking at altitude or in mountaineering. Also, some endurance sports involve high-intensity exercise in hypoxia, such as mountain stages in cycling (up to ~2,800 m altitude in Tour de France) or mountain running events (i.e., Pikes Peak marathon to ~4,300 m in Colorado). It is well known that the drop in arterial pO₂ occurring at altitude impairs exercise tolerance. This ergolytic exercise-induced increase in [O₂Hb] was lower in HYPCON (F₁ = 28.8, P < 0.05). Correspondingly, Δ[HHb] was higher in HYPCON than in NORCON (F₁ = 43.3, P < 0.05). However, Δ[O₂Hb] and Δ[HHb] were similar between HYPCON and HYPBR at all times. EX₄₅% increased [THb] to the same degree in all conditions, yet during EXmax, the exercise-induced increase in [THb] was greater in NORCON than in HYPCON (P < 0.05).

RPE, AMS, and blood pressure. In EX₄₅%, the Borg scores were higher in HYPCON (13.6 ± 0.7) and HYPBR (13.3 ± 0.5) than in NORCON (10.1 ± 0.4, P < 0.05). Conversely, at EXmax, Borg scoring was identical among the three conditions (NORCON: 18.6 ± 0.3; HYPCON: 18.3 ± 0.3; HYPBR: 18.5 ± 0.3). AMS scores were higher in HYPCON (2.5 ± 0.6; range 0–8) than in NORCON (0.8 ± 0.2; range 0–2, P < 0.05), but there was no significant difference between HYPCON and HYPBR (2.5 ± 0.5; range 0–6). Still, it is worthwhile to mention that six out of 10 subjects in HYPCON vs. only four in HYPBR were diagnosed positive for AMS (χ² = 5.0, P = 0.99). Systolic blood pressure was similar between NORCON (126 ± 2 Torr) and HYPCON (129 ± 3 Torr). However, in HYPBR, systolic pressure was reduced slightly (121 ± 2 Torr, P < 0.05). Diastolic blood pressure, on average, was 69 ± 1 Torr and was independent of the experimental conditions.

Fig. 5. Effect of nitrate supplementation in hypoxia on oxygenation status in the prefrontal cortex at rest and during exercise. The typical time course of TOI, measured by NIRS throughout an experimental session, is presented for a representative subject (A). Mean ± SE (n = 15; B) was calculated from the shaded time windows in A for rest and constant-load exercise at EX₄₅%, as well as for the last 30 s of the EXmax in NORCON (○), HYPCON (■), and HYPBR (●). Subjects performed 1 experimental session in normoxia (○) and 1 session in 11% ambient O₂ content (■, ●) in the presence (●) or absence (○, ■) of dietary nitrate supplementation. See MATERIALS AND METHODS for further details. *P < 0.05 compared with NORCON.
effect is primarily due to inhibition of oxidative energy production in active muscles, but at higher altitudes, the impact of AMS also comes into prominence. Therefore, any intervention that could improve whole-body O2 efficiency may stimulate exercise tolerance in hypoxia even more than in normoxia. Against this background, we investigated the effect of dietary nitrate supplementation in the form of BR on muscle and cerebral oxygenation status during submaximal and maximal exercise at simulated high altitude. Subjects were acutely exposed to an exercise protocol involving submaximal and maximal exercise in 11% ambient O2. This elicited the expected physiological responses, such as decreased arterial pO2 and %SpO2, along with exaggerated pulmonary VE, decreased muscle and cerebral oxygenation status, elevated lactate production during submaximal exercise, as well as development of AMS symptoms. Compared with normoxia, maximal exercise capacity in hypoxia was also markedly reduced. We hypothesized that supplementary nitrate intake, by reducing the O2 cost of muscle contractions, could 1) improve muscle oxygenation status for a given exercise intensity, 2) prevent AMS symptoms by suppressing the exercise-induced drop in arterial pO2, and 3) via both of these mechanisms, improve exercise capacity. The data clearly demonstrate that nitrate administration beneficially impacted on muscle oxygenation status during both submaximal and maximal exercise in hypoxia. Exercise capacity was concomitantly enhanced. However, neither cerebral oxygenation status nor AMS symptoms were significantly affected by the nitrate supplementation.

Recent studies have shown unanimously that dietary nitrate supplementation can reduce the rate of VO2 for a given workload during submaximal exercise (2, 3, 33, 35, 36, 50). In the present study, subjects performed a 20-min constant-load exercise bout at a workload of ~45% of sea-level VO2peak (EX45%), corresponding with ~70% of the VO2peak measured at simulated 5,000 m altitude. As expected, hypoxia per se did not alter whole-body VO2 during this submaximal exercise. However, BR ingestion suppressed VO2 by ~4%, which probably largely reflects a reduced rate of VO2 by active muscles. Thus TOI in m. vastus lateralis was higher in HYPBR than in HYPCON (Fig. 4), along with a higher fraction of circulating oxygenated hemoglobin (%SpO2; Fig. 3) and lower exercise-induced increase in muscle deoxygenated hemoglobin content (Δ[HHb]; Table 3), indicating a lower rate of muscle O2 extraction. The lower O2 cost of EX45% also translated into a tendency of an improved mechanical efficiency of cycling (~5% increase in net NE, P = 0.07) and lower blood lactate accumulation (Fig. 1). Taken together, our above findings prove that the effect of acute severe hypoxia to inhibit oxidative energy production in muscles during submaximal exercise is suppressed by dietary nitrate supplementation. This beneficial effect is probably at least partly explained by better coupling of mitochondrial respiration with oxidative phosphorylation (elevated phosphate/O2 ratio) (34), as well as by a reduced ATP cost for a given rate of muscle contractions (2).

Contrary to our expectations, the effect of BR on VO2 was not greater than for similar nitrate supplementation protocols during exercise in normoxia (2, 3, 33, 35, 36, 50). In this regard, it is important to note that here, we only looked at the effects of an acute bout of severe hypoxia exposure with no prior acclimatization. It has been demonstrated that altitude acclimatization per se can enhance O2 efficiency during submaximal exercise (41). Therefore, it is tempting to speculate that long-term altitude acclimatization in conjunction with nitrate supplementation might yield additive effects on O2 efficiency during exercise.

One would expect a more efficient oxidative energy production due to nitrate intake also to enhance maximal exercise performance at altitude. Therefore, we also investigated the effects of nitrate supplementation on performance in an EXmax. Hypoxia per se reduced time to exhaustion by ~35%, which is in line with earlier observations at ~5,000 m altitude (12, 53), and BR administration eliminated ~5% of this ergolytic effect (Fig. 2). Conversely, Vanhatalo et al. (51) recently reported BR to increase time to exhaustion by >15% during an ~5- to 10-min constant-load maximal knee-extension test in hypoxia (14.5% ambient O2, ~3,000 m altitude). However, during exercise with a small muscle group in moderate hypoxia (51), improved muscular O2 efficiency is likely to translate directly into improved performance, because muscles are overperfused. Thus mitochondrial O2 use, rather than muscle O2 delivery, is directly limiting muscle VO2. In contrast, during whole-body exercise in severe hypoxia, VO2 in active muscles is primarily limited by impaired O2 diffusion due to drop of arterial pO2 (Fig. 3) (53). Furthermore, maximal constant-load tests (51) are often more sensitive than maximal incremental exercise protocols to detect changes in exercise capacity (56). Moreover, at high altitude, but not at moderate altitude (51), AMS becomes a primary determinant of exercise tolerance. Accordingly, in HYPCON, six out of 15 subjects developed AMS. In these specific subjects, compared with NORCON, HRpeak dropped by 24 ± 4 beats/min, whereas in the others, it dropped by only 14 ± 3 beats/min (P < 0.05). This indicates that maximal exercise capacity was limited by AMS symptoms indeed.

Interestingly, the effect of BR to boost maximal exercise capacity in hypoxia (Fig. 2) occurred against the face of unchanged VO2peak (Table 2). This finding is in line with earlier studies in normoxia, showing improved maximal exercise capacity at constant VO2max (2, 3, 50). This is probably at least partly explained by facilitated oxidative energy turnover throughout the incremental exercise, allowing for better maintenance of high-energy phosphate content and suppression of inorganic phosphate and hydrogen ion accumulation (2, 51). Such a mechanism is likely to inhibit the development of local muscle fatigue in the final stages of a EXmax. Support for greater O2 efficiency during EXmax comes from our observation that TOI in m. vastus lateralis was higher in HYPBR than in HYPCON (Fig. 4). This was associated with smaller Δ[HHb] (Table 3) and occurred against the face of equal O2 delivery, as indicated by similar Δ[THb] (Table 3), as well as %SpO2 (Fig. 3). Accordingly, BR intake in patients affected by peripheral arterial disease decreased muscle O2 extraction in areas of hypoxia, improved tissue oxygenation, as well as enhanced exercise tolerance (31). Further along this line, BR was also found to stimulate muscle phosphocreatine resynthesis postexercise in hypoxia (51), which is probably at least partly explained by enhanced blood flow and O2 delivery to the more hypoxic muscle areas of the muscle and thereby, improved oxidative ATP production (26, 30, 48).

It has been proposed that increased NO production due to nitrate supplementation may also stimulate exercise performance by promoting muscle blood flow during exercise (25).
In keeping with earlier studies in normoxia (3, 32, 33), we found that nitrate intake in hypoxia reduced resting systolic blood pressure, conceivably indicating decreased total peripheral vascular resistance. We did not measure blood pressure during exercise but estimated changes in muscle blood flow during exercise via NIRS. \( \Delta[THb] \), which is the sum of \( \Delta[O2Hb] \) and \( \Delta[HHb] \) (Table 3), as a rule correlates well with changes in tissue blood flow (49). \( \Delta[THb] \) was not affected by BR supplementation either during EX45\% and/or during EXmax. Thus if nitrate intake were successful to increase NO production in muscles during exercise, this clearly did not impact on exercise-induced stimulation of blood flow. It has been previously reported that acute exposure to 10.5%-inspired O2 content (~5,300 m altitude) slightly increased muscle blood flow during submaximal but not during maximal cycling exercise (12). In contrast, we found acute exposure to 11% ambient O2 (NORCON vs. HYPCON) not to alter \( \Delta[THb] \) during submaximal exercise, indicating unchanged blood flow. In this regard, it is important to note that \( \Delta[THb] \) obtained from NIRS is an indirect, semiquantitative measure of blood flow, and therefore, data are normalized relative to the baseline signal (rest). Hence, similar \( \Delta[THb] \) does not exclude that absolute rates of blood flow in EX45\% were still higher in HYPCON than in NORCON due to higher blood flow existing already at rest. However, in the aforementioned study, resting muscle blood flow was not reported (12). Finally, it has also been suggested by some that NO might contribute to facilitating oxidative energy production during the transition from rest to exercise by stimulating local vasodilation and O2 delivery to muscle cells (14). However, the slopes of \( \Delta[THb] \) at the onset of either EX45\% or EXmax were identical between HYPCON and HYPBR, indicating that such vascular effect was probably unimportant.

Another hypothesis driving the current study was that nitrate administration could enhance exercise capacity at altitude by preventing central mechanisms limiting exercise performance. It is the prevailing opinion that the drop of cerebral O2 delivery, which also results in increased formation of reactive O2 species (1), plays an important role in the development of AMS and high-altitude cerebral edema (HACE), which impair exercise tolerance (28, 52). Therefore, any intervention able to improve cerebral oxygenation status conceivably could stimulate exercise performance at high altitude. Here, we measured cerebral oxygenation status in the prefrontal cortex using NIRS. In keeping with literature data (28, 52), basal oxygenation status (TOI) in the prefrontal cortex was lower in hypoxia than in normoxia (Fig. 5). Furthermore, TOI decreased substantially from rest to submaximal exercise (EX45\%) with only a minor, further drop occurring during EXmax. However, BR administration clearly did not alter cerebral oxygenation status or blood flow, as evidenced by similar prefrontal cortex TOI, \( \Delta[O2Hb] \), \( \Delta[HHb] \), and \( \Delta[THb] \) values in HYPCON and HYPBR during both EX45\% and EXmax. In addition, BR did not consistently inhibit the development of AMS. This may reflect the fact that physiological responses to acute hypoxia by definition are probably primarily targeted to protect the central nervous system from hypoxic damage (13, 44), which makes exogenous stimulation of NO synthesis by nitrate ingestion redundant. In addition, autoregulation of cerebral blood flow is only abolished once \%SpO2 drops below 60% (43). This threshold was not exceeded in the conditions of the present study, either at rest or during exercise (\%SpO2 > 60%; Fig. 3), which concurs with earlier observations (12, 53). Nonetheless, it would be premature to conclude from the present findings that oral nitrate is ineffective to inhibit the development of AMS. First, it is the prevailing opinion that the drop of arterial O2 content occurring in hypoxia is the primary trigger of AMS (5). Interestingly, we here for the first time demonstrate that oral nitrate supplementation can increase the fraction of oxygenated arterial hemoglobin (%SpO2) both at rest and during submaximal exercise. It is well documented that a drop of alveolar \( \rho \)O2 limits pulmonary O2 diffusion capacity at high altitude, which results in a drop of %SpO2 (13, 46). In the face of impaired pulmonary O2 diffusion, the rate of peripheral O2 use becomes an important determinant of circulating arterial O2 content. Therefore, the effect of nitrate ingestion to improve peripheral O2 efficiency (Table 2 and refs. (2, 3, 33, 35, 36, 50)) should increase %SpO2, particularly because oxyhemoglobin saturation in severe hypoxia is operating on the steep segment of the oxyhemoglobin dissociation curve. Second, the hypoxic stimulus imposed to our subjects was moderate (~2 h at ~5,000 m), which limited the incidence as well as the severity of AMS symptoms. The potential of nitrate supplementation to prevent AMS by increasing %SpO2 may become more pertinent during a more severe hypoxic challenge at extreme altitude (>5,500 m). In fact, the small arterial [O2Hb] desaturation induced by short exposure to 11% ambient O2 would be largely negated by short-term acclimatization (13). Finally, oxygenation status measured at the site of the prefrontal cortex may not be representative for oxygenation status in other brain areas that play a more prominent role in the pathogenesis of AMS, such as the temporal region (21).

By analogy with other recent studies, we have used BR juice as a vehicle for nitrate supplementation. However, BR juice contains many substances other than nitrate, including betaine, antioxidants, and polyphenols, which also could affect physiological responses. However, studies with denitrificated BR juice have indicated that the known physiological effects of BR consumption, at least in normoxia, are mainly if not entirely accounted for by its high nitrate content (32, 33). In keeping with earlier observations, in the conditions of the current study, BR raised plasma nitrate concentration by a factor ~3.5, which resulted in a 40% increment in plasma nitrite (Table 1). This effect was clearly ample to enhance muscular O2 efficiency during exercise in hypoxia. Still, the rise in plasma nitrate and nitrite was smaller than in other studies using similar nitrate dosages in young, healthy volunteers (2, 3, 32, 33, 35, 36). This is probably, at least partly, explained by the fact that we administered BR in small aliquots over a 1-h period, whereas others used a bolus (2, 3, 32, 33, 35, 36), which conceivably resulted in lower peak plasma levels. In addition, blood samples were taken 2 h after the last BR aliquot, which is before peak plasma nitrite levels normally occur (55). Plasma nitrite concentrations were approximately tenfold higher than in most other recent studies looking at the effects of nitrate supplementation in young, healthy subjects indeed (2, 3, 32, 33, 50, 51) yet were within the range of basal plasma nitrite values reported in literature (24, 29).

In conclusion, oral nitrate supplementation improves muscle oxygenation status during both submaximal and maximal exercise in severe acute hypoxia. This is associated with enhanced exercise capacity. In the conditions of the present study, nitrate intake did not inhibit a hypoxia-induced drop of
cerebral oxygenation or the development of AMS. However, the fraction of oxygenated arterial hemoglobin was increased significantly at rest, as well as during submaximal exercise. It remains to be investigated whether muscle O2 sparing, due to nitrate intake during prolonged exposure to high altitude, can elevate arterial O2 content to such a degree that prevention of AMS and HACE becomes an additional mechanism for improvement of exercise tolerance.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: E.M. and P.H. conception and design of research; E.M. performed experiments; E.M., X.W., and A.V.S. analyzed data; E.M., R.V.T., and P.H. interpreted results of experiments; E.M. prepared figures; E.M. and P.H. drafted manuscript; R.V.T., X.W., A.V.S., M.T., and P.H. edited and revised manuscript; E.M., R.V.T., X.W., A.V.S., M.T., and P.H. approved final version of manuscript.

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