No effect of dietary nitrate supplementation on endurance training in hypoxia

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We investigated whether dietary nitrate (NO_3^-) supplementation enhances the effect of training in hypoxia on endurance performance at sea level. Twenty-two healthy male volunteers performed high-intensity endurance training on a cycle ergometer (6 weeks, 5×30 min/week at 4–6 mmol/L blood lactate) in normobaric hypoxia (12.5% FiO_2), while ingesting either beetroot juice [0.07 mmol NO_3^-/kg body weight (bw)/day; BR, $n = 11$] or a control drink (CON, $n = 11$). During the pretest and the posttest, the subjects performed a 30-min simulated time trial (TT) and an incremental $\text{VO}_{2\text{max}}$ test. Furthermore, a biopsy was taken from m. vastus lateralis before and after the TT. Power output during the training sessions in both

groups increased by ~6% from week 1 to week 6 ($P < 0.05$). Compared with the pretest, $\text{VO}_{2\text{max}}$ in the posttest was increased ($P < 0.05$) in CON (5%) and BR (9%). Power output corresponding with the 4 mmol/L blood lactate threshold, as well as mean power output during TT increased by ~16% in both groups ($P < 0.05$). Muscle phospho-AMP-activated protein kinase, hypoxia inducible factor-1 α mRNA content, and glycogen breakdown during the TT were similar between the groups in both the pretest and the posttest. In conclusion, low-dose dietary NO_3^- supplementation does not enhance the effects of intermittent hypoxic training on endurance exercise performance at sea level.

Altitude training is a popular ingredient in the training plan of endurance athletes preparing for sea-level competition. Different protocols for altitude training have been developed. However, “living high – training low” remains to be the most popular model because beneficial physiological effects of altitude exposure are obtained while maintaining normal training intensity during sea-level training sessions (Millet et al., 2010). The development of hypoxicators allowing to create home-based spaces in normobaric hypoxia for either rest or training, has added novel degrees of freedom to develop variable altitude training protocols to beneficially impact on sea-level performance. In this regard, over the last 10–15 years, interest in the potential of intermittent hypoxic training (IHT), while living at sea level, on functional performance in normoxia, has grown. Most studies have shown beneficial physiological adaptations in skeletal muscle due to IHT, including increased capillary and mitochondrial density and elevated glycolytic and oxidative enzyme expression (Geiser et al., 2001; Vogt et al., 2001; Zoll et al., 2006; Hoppeler et al., 2008). We recently also reported a 6-week period of intermittent sprint training in hypoxia to elevate muscle phosphofructokinase activity compared with similar training in normoxia (Puype et al., 2013). Nonetheless, while IHT seems to induce favorable myocellular adaptations, the effects on functional capacity, measured as either $\text{VO}_{2\text{max}}$

or endurance performance at sea level, are equivocal (Hoppeler et al., 2008; Bonetti & Hopkins, 2009; Millet et al., 2010).

There is also evidence to indicate that nitric oxide (NO) is implicated in human adaptation to hypoxia (Levett et al., 2011; Limberg et al., 2011). NO is a ubiquitous signaling molecule playing an important role in a wide variety of physiological processes in different tissues in the human body, including skeletal muscle (Stamler & Meissner, 2001). Literature data indicate that enhanced NO production may be an important factor in human adaptation to altitude. Accordingly, well-acclimatized Tibetan highlanders exhibit elevated circulating plasma nitrite (NO_2^-) levels, which conceivably reflects an upregulation of NO synthase (NOS) activity (Erzurum et al., 2007a). Besides this endogenous pathway, NO can also be derived via the sequential reduction of dietary nitrate (NO_3^-) to NO_2^- , and further to NO. Furthermore, recent studies have demonstrated that oral NO_3^- intake, either as pure sodium NO_3^- or in the form of beetroot juice (BR), enhanced endurance exercise performance (Lansley et al., 2011a; Bond et al., 2012; Cermak et al., 2012) or exercise capacity (Bailey et al., 2009, 2010; Vanhatalo et al., 2010, 2011; Lansley et al., 2011b; Masschelein et al., 2012) in young healthy volunteers. This ergogenic effect is at least partly explained by improved mitochondrial oxygen

(O₂) efficiency (Larsen et al., 2011, 2012). O₂ efficiency is particularly important in any condition that is associated with limited O₂ availability, such as exercise at altitude. Moreover, increased NO-mediated vasodilation and muscle blood flow (Maiorana et al., 2003; Erzurum et al., 2007b; Umbrello et al., 2013) by facilitating myocellular O₂ extraction, may also play a role in improving exercise performance in hypoxia. Interestingly, studies in our and other laboratories found lower rate of O₂ consumption for a given rate of submaximal exercise (Masschelein et al., 2012; Muggeridge et al., 2014), as well as improved exercise tolerance (Vanhatalo et al., 2011; Masschelein et al., 2012) in hypoxia due to NO₃⁻ intake.

Against this background, it is reasonable to postulate that dietary NO₃⁻ supplementation may be a useful strategy to facilitate physiological adaptations to exercise training in hypoxia. Therefore, in this study, we compared the effects of 6 weeks of intermittent high-intensity endurance training in hypoxia, alone or in conjunction with dietary NO₃⁻ supplementation, on maximal exercise capacity and endurance exercise performance at sea level.

Methods

Subjects

Twenty-two healthy, male subjects, aged 18–30 years volunteered to take part in the study, which was approved by the KU Leuven Ethics Committee. Subjects gave their written informed consents after they were informed of all experimental procedures and risks associated with the experiments, and underwent a medical screening. Exclusion criteria for participation were smoking, recent exposure to hypoxia, and risk factors that could compromise the subject's safety during the study. Subjects were asked to continue their normal sports and other physical activities throughout the study, and they were instructed to refrain from heavy exercise, alcohol, and caffeine-containing drinks for 48 h prior to the experimental sessions. Furthermore, they received a list of NO₃⁻-rich foods to be avoided during the entire study period, aiming to reduce involuntary daily NO₃⁻ intake. Subjects were also instructed to refrain from using antibacterial mouthwash products, which would inhibit the conversion of NO₃⁻ to NO₂⁻ by bacteria in the oral cavity (Govoni et al., 2008).

Experimental protocol

Study design and experimental groups

A randomized controlled study was performed. Subjects participated in a 6-week training program consisting of high-intensity endurance training, with a pretest before and a posttest following the intervention. One week before the pretest, a venous blood sample (10 mL) was taken from an arm vein into lithium heparin tubes (BD Vacutainer, Franklin Lakes, New Jersey, USA). Plasma was immediately separated by centrifugation (2700 g for 10 min at 4 °C) and frozen at –80 °C for later analysis of [NO₃⁻]. The subjects also performed an incremental VO_{2max} test (60 W + 20 W/min) on a bicycle ergometer (Avantronic Cyclus 2, Leipzig, Germany). Subsequently, they were randomly assigned to either of two experimental groups with similar distributions for age, body weight (bw), and VO_{2max} (Table 1). Both groups were enrolled in a supervised endurance training program for 6 weeks. However, one

Table 1. Subject characteristics

	CON	BR
Age (yr)	21.4 ± 0.8	21.7 ± 0.6
Weight (kg)	72.0 ± 2.7	72.3 ± 4
VO _{2max} (mL/min/kg)	60.8 ± 1.8	60.1 ± 2.7

Values are means ± SEM. Subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}) while receiving either NO₃⁻ supplementation in the form of beetroot juice (BR, *n* = 11) or a control drink (CON, *n* = 11).

group received a control drink during this period (CON, *n* = 11), while the other group received beetroot juice (BR; *n* = 11). Following the randomization, the subjects participated in two familiarization sessions with a 24-h interval. In each session, they performed a 30-min simulated time trial (TT) on the cycle ergometer. Mean power output registered in the latter session was used as the initial workload for the 30-min TT performed during both the pretest and the posttest (see later). Four days before the pretest, the subjects started the supplementation of either NO₃⁻ (0.07 mmol/kg bw/day) in the form of BR (700 mg NO₃⁻/L), or an equivalent volume of apple-blackcurrant juice (Pajottenlander, Pepingen, Belgium), which served as a control drink. The protein, carbohydrate, fiber, and vitamin content of the control drink was comparable to BR, yet containing negligible amounts of NO₃⁻. The daily dose was distributed over three equal aliquots to be ingested with breakfast, lunch, and dinner, respectively. However, during the pretest and the posttest, the full daily dose of either BR (0.07 mmol NO₃⁻/kg bw, ~500 mL) or the control drink was administered between 2.5 and 2 h before the experimental session (TT) started. This supplementation was continued till the end of the posttest, which was identical to the pretest. The subjects were not informed of the exact composition of the juices and they were blinded to the true experimental hypotheses till the end of the study.

Training intervention

The subjects trained in a normobaric hypoxic facility at 12.5% F_{O2}, corresponding to ~4000-m altitude. The training protocol was adapted from Vogt et al. (2001). Subjects participated for 6 weeks in five supervised training sessions per week on a bicycle ergometer. Blood lactate during the 30-min training sessions was measured at 10-min intervals, and whenever needed, workload was immediately adjusted to obtain steady-state blood lactate concentrations between 4 and 6 mmol/L (Vogt et al., 2001) while cycling at cadences between 80 and 100 rpm. Each session started with a 9-min warming up (3 min at 70%, 3 min at 80%, and eventually 3 min at 90% of the training workload). The sessions ended with a 10-min cooling down at 50 W. During each session, heart rate was continuously registered (Polar, Kempele, Finland), and a capillary blood sample (5 µL) was collected from the earlobe at 10 and 20 min for control of blood lactate concentration (Lactate Pro, Arkray, Japan).

Pretest and posttest

The pretest and the posttest each involved two experimental sessions interspersed by a 2-day rest interval. On the evening before each test day, the subjects received a standardized dinner (1000 kcal: 60E% carbohydrates, 27E% fat, 13E% protein). In the first experimental session, the subjects reported to the laboratory between 7:00 and 10:30 h after a 10- to 12-h overnight fast. On arrival, they received a standardized carbohydrate-rich breakfast (710 kcal: 84E% carbohydrates, 7E% fat, 9E% protein) together

with BR (0.07 mmol NO₃⁻/kg bw) or CON. After a ~2-h rest, a venous blood sample (10 mL) was taken from an arm vein into lithium heparin tubes. Furthermore, a percutaneous needle biopsy was taken from the left m. vastus lateralis under local anesthetic (2–3 mL lidocaine, subcutaneously) through an incision in the skin. Following the biopsy, the subjects started the 30-min TT session on the cycle ergometer. After a 10-min warming up (5 min at 100 W plus 5 min at 150 W) the TT was started (t_0 – t_5) at 198 ± 4 W, which is the individual workload obtained from the second familiarization session. From t_5 to t_{25} , subjects could adjust the workload at 5-min intervals according to their subjective perception of fatigue. From t_{25} to t_{30} , 1-min adjustments were allowed so as to reach a point of full exhaustion at the end of the TT. Because workload was set identical between the pretest and the posttest from t_0 to t_5 , values measured in this time window were not further considered in the statistical analyses (see later). At t_0 , t_{20} , and t_{30} , a 5- μ L blood sample was taken from the earlobe for lactate determination. Arterial O₂ saturation (%SpO₂) was measured by pulseoxymetry using a sensor just above the left eyebrow (Nellcor N-600-x, Oxismart, Mallinckrodt, St. Louis, Missouri, USA). Furthermore, heart rate and power output were continuously measured but were hidden to the subjects. At the end of the TT, a second muscle biopsy was taken through the same incision as the preexercise biopsy. Two days later, the subjects returned to the laboratory between 7:00 and 10:30 h after a 10- to 12-h overnight fast to perform a VO_{2max} test. Initial workload was set at 60 W and was increased by 35 W/3 min until volitional exhaustion or failure to maintain cadence > 65 rpm. During the test heart rate, %SpO₂ and pulmonary gas exchange parameters (Cortex® Metalyzer II, Leipzig, Germany) were continuously measured and blood (5 μ L) was sampled from the earlobe for lactate assay at the end of each stage. Following the 6-week intervention period, subjects abstained from training for 3 days, where after they participated in the posttest, which was identical to the pretest and on the same days of the week. The BR and CON were supplied till the last day of the posttest.

Analysis of blood and muscle samples

Plasma nitrate

Blood sampling for NO₃⁻ assay was only included in a later stage of the study, yielding a reduced number of observations (CON, $n = 7$; BR, $n = 6$). Following sampling, the plasma was immediately separated by centrifugation and stored at -80°C for later analysis of [NO₃⁻] by a fluorometric assay using 2,3-diaminonaphthalene (Nussler et al., 2006).

Muscle biochemistry

Part of the muscle samples was immediately frozen in liquid nitrogen and stored at -80°C till later analysis. Muscle glycogen content was measured as glucose residues after acid hydrolysis using a standard enzymatic fluorometric assay (Lowry & Passoneau, 1972).

Western blotting

Frozen muscle tissue (~20 mg) was homogenized 3×5 s with a Polytron mixer in ice-cold buffer (1:10, w/v) [50-mM Tris-HCl pH 7.0, 270-mM sucrose, 5-mM EGTA, 1-mM EDTA, 1-mM sodium orthovanadate, 50-mM glycerophosphate, 5-mM sodium pyrophosphate, 50-mM sodium fluoride, 1-mM DTT, 0.1% Triton-X 100, and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were then centrifuged at 10 000 g for 10 min at 4°C . The supernatant was collected and immediately stored at -80°C . The protein concen-

tration was measured using the DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium). Thirty to eighty micrograms of proteins were separated by SDS-PAGE (10% gel) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% nonfat milk for 1 h and afterwards incubated overnight (4°C) with the following antibodies (1:1000, Cell Signaling, Leiden, The Netherlands): phospho-AMP-activated protein kinase (AMPK) and total AMPK. Horseradish peroxidase-conjugated antirabbit secondary antibody (1:5000) (Sigma-Aldrich, Bornem, Belgium) was used for chemiluminescent detection of the proteins. Membranes were scanned and quantified with Genesnap and Genetools Softwares (Syngene, Cambridge, UK), respectively (Deldicque et al., 2010). Results were expressed relative to a standard sample that was run together with the samples to allow comparisons between membranes, and phospho-AMPK data are reported relative to AMPK total. All values were divided by the mean value of the first biopsy sample (pretest) from the respective condition.

RNA extraction, reverse transcription and real-time qPCR analysis

Total RNA was extracted using TRIzol (Invitrogen, Vilvoorde, Belgium) from 20 to 25 mg of frozen muscle tissue. RNA quality and quantity were assessed by spectrophotometry with a Nanodrop (Thermo Scientific, Erembodegem, Belgium). One microgram of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Gent, Belgium) according to manufacturer's instructions. A SYBR Green-based Master Mix (Applied Biosystems) was used for real-time polymerase chain reaction (PCR) analyses using the ABI PRISM 7300 (Applied Biosystems). Real-time PCR primers were designed for hypoxia inducible factor-1 α (HIF-1 α ; primer sequence: forward, GCC CCA GAT TCA GGA TCA GA; reverse TGG GAC TAT TAG GCT CAG GTG AAC). Thermal cycling conditions consisted of 40 three-step cycles including denaturation of 30 s at 95°C , annealing of 30 s at 58°C , and extension of 30 s at 72°C . All reactions were performed in triplicate. To compensate for variations in input RNA amounts and efficiency of reverse transcription, ribosomal protein L4 (RPL4), RPL19, and beta-2-microglobulin (β -2-MG) mRNA were quantified and results were normalized to these values. These genes were chosen out of five normalization genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere (Vandesompele et al., 2002). All values were divided by the mean value of the first biopsy sample (pretest) from the respective condition.

Statistical analyses

The effect of the intervention was evaluated using repeated-measures analysis of variance (ANOVA). Two-way ANOVA was performed to compare the effects of the training in hypoxia in the absence or presence of daily NO₃⁻ intake (CON vs BR). A planned contrast analysis was used for post hoc comparisons when appropriate. Contrast analysis was also used to evaluate specific preplanned comparisons. Also, the 95% confidence intervals [mean $\pm$ 1.96 $\times$ standard error of mean (SEM)] of the relative change in performance parameters were calculated. A probability level (P) ≤ 0.05 was considered statistically significant. All data are expressed as means $\pm$ SEM.

Results

Training intensity (Table 2)

Mean power output, heart rate, and blood lactate were similar between CON and BR throughout the training

Table 2. Training intensity at the start and at the end of the training period

	Week 1	Week 6
Mean power output (Watt)		
CON	126 ± 6	134 ± 5*
BR	139 ± 6	147 ± 5*
Heart rate (bpm)		
CON	165 ± 2	165 ± 2
BR	160 ± 2	160 ± 2
% HR max		
CON	83 ± 1.0	83 ± 0.8
BR	86 ± 1.5	86 ± 1.2
Blood lactate (mmol/L)		
CON	5.68 ± 0.19	5.75 ± 0.18
BR	5.59 ± 0.27	5.23 ± 0.14

* $P < 0.05$ vs week 1.

Values are means ± SEM. Subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}) while receiving either NO₃⁻ supplementation in the form of beetroot juice (BR, $n = 11$) or a control drink (CON, $n = 11$). Mean power output, heart rate (HR), and blood lactate were measured after 20 min in the 30-min training bouts during week 1 and week 6 of the training period. Percent HR max is expressed relative to the corresponding maximal HR measured in the pretest (Table 4).

period. Mean power outputs during training increased by ~6% in both groups from week 1 to week 6 ($P < 0.05$).

TT performance (Table 3)

In the pretest, there were no significant differences between the groups either for mean power output, or for heart rate or blood lactate concentration during the TT. However, %SpO₂ was slightly higher in BR than in CON ($P < 0.05$). From the pretest to the posttest, mean power

Table 3. Effects of beetroot juice in conjunction with high-intensity endurance training in the time trial

	Pretest	Posttest	Δ (CI)
Mean power output(Watt)			
CON	203 ± 14	235 ± 14*	32 (28; 36)
BR	215 ± 8	252 ± 9*	37 (28; 45)
Mean SpO ₂ (%)			
CON	96.6 ± 0.4	97.2 ± 0.2	0.5 (0.1; 1.0)
BR	98.1 ± 0.2†	97.8 ± 0.3	-0.2 (-0.9; 0.4)
Mean HR (bpm)			
CON	182 ± 3	183 ± 4	0.7 (-5; 7)
BR	177 ± 4	176 ± 4	-0.3 (-3; 3)
% HR max			
CON	92 ± 1.0	93 ± 1.6	0.9 (-2.9; 4.8)
BR	95 ± 1.4	95 ± 1.0	-0.1 (-1.6; 1.4)
Lactate at 20 min (mmol/L)			
CON	6.8 ± 0.5	7.2 ± 0.6	0.4 (-0.5; 1.2)
BR	5.2 ± 0.7	5.6 ± 0.7	0.4 (-1.0; 1.8)
Lactate at 30 min (mmol/L)			
CON	8.5 ± 0.6	9.5 ± 0.6	1.0 (-0.03; 2.0)
BR	8.0 ± 0.5	9.3 ± 0.5	1.3 (-0.4; 3.0)

* $P < 0.05$ vs pretest; † $P < 0.05$ vs CON.

Values are means ± SEM, except for Δs where mean and 95% confidence intervals (CIs) are given. Subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}) while receiving either NO₃⁻ supplementation in the form of beetroot juice (BR, $n = 11$) or a control drink (CON, $n = 11$). In both the pretest and the posttest, the subjects performed a 30-min simulated time trial on a cycle ergometer. HR, heart rate; % HR max is expressed relative to the corresponding maximal HR measured in the pretest (Table 4).

output during the TT increased to the same extent (~17%) in CON and BR ($P < 0.05$). Also, %SpO₂, heart rates, as well as blood lactate levels in the posttest, were similar between the groups.

Incremental exercise test (Table 4)

Irrespective of the experimental condition, the training intervention increased time to exhaustion from ~22.5 min in the pretest to ~25.5 min in the posttest [$P < 0.05$ vs pretest; non-significant (n.s.) between groups]. VO_{2max} increased by ~5% in CON vs ~9% in BR ($P < 0.05$ vs pretest; n.s. between groups). Peak heart rates were similar between the pretest and the posttest, yet were slightly lower in BR than in CON ($P < 0.05$). Furthermore, blood lactate and respiratory exchange ratio at peak exercise were similar between the pretest and the posttest in both groups, yet in BR, peak blood lactate was slightly lower in the posttest than in the pretest ($P < 0.05$). Furthermore, the training increased power output at the 4-mM lactate threshold (LT4) by ~13% in CON vs ~16% in BR ($P < 0.05$ vs pretest; n.s. between groups). Irrespective of the experimental condition, %SpO₂ values at peak exercise (data not shown) were in the range of 94–96% in both the pretest and the posttest.

Muscle glycogen, phospho-AMPK and HIF-1α mRNA expression (Table 5)

Muscle biopsies were taken before and immediately after the 30-min TT in the pretest and the posttest. There were no significant differences between the groups either

Table 4. Effects of beetroot juice in conjunction with high-intensity endurance training during the incremental VO_{2max} test

	Pretest	Posttest	Δ (CI)
Power output at LT4 (Watt)			
CON	204 ± 12	231 ± 10*	27 (16; 38)
BR	215 ± 10	249 ± 11*	34 (27; 42)
Time to exhaustion (s)			
CON	1346 ± 63	1505 ± 52*	158 (128;189)
BR	1371 ± 47	1548 ± 53*	177 (136; 219)
VO _{2max} (mL/min/kg)			
CON	60.8 ± 1.8	63.8 ± 1.6*	3.0 (0.1; 5.9)
BR	60.1 ± 2.6	65.6 ± 2.1*	5.5 (3.2; 7.8)
Peak heart rate (beats/min)			
CON	198 ± 2	197 ± 2	-1.3 (-4.1; 1.5)
BR	188 ± 3†	186 ± 3†	-0.8 (-4.4; 2.8)
Peak blood lactate (mmol/L)			
CON	12.4 ± 0.5	11.8 ± 0.4	-0.7 (-1.6; 0.3)
BR	11.9 ± 0.7	10.4 ± 0.7*	-1.5 (-2.5; -0.5)
Peak RER			
CON	1.12 ± 0.02	1.10 ± 0.02	-0.01 (-0.04; 0.02)
BR	1.12 ± 0.02	1.11 ± 0.02	-0.02 (-0.06; 0.02)

* $P < 0.05$ vs pretest; † $P < 0.05$ vs CON.

Values are means ± SEM, except for Δs where mean and 95% confidence intervals (CIs) are given. Subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}) while receiving either NO₃⁻ supplementation in the form of beetroot juice (BR, $n = 11$) or a control drink (CON, $n = 11$). In both the pretest and the posttest, the subjects performed a maximal incremental exercise test on a cycle ergometer. RER, respiratory exchange ratio.

Table 5. Effects of beetroot supplementation in conjunction with high-intensity endurance training on muscle biochemistry before and after a time trial

	CON		BR	
	Before	After	Before	After
Glycogen content				
Pretest	142 ± 12	73 ± 8 [†]	156 ± 13	94 ± 13 [†]
Posttest	185 ± 17*	103 ± 11* [†]	185 ± 10*	125 ± 11* [†]
HIF1- α mRNA				
Pretest	1.00 ± 0.17	1.08 ± 0.08	1.00 ± 0.08	1.08 ± 0.15
Posttest	1.44 ± 0.08	1.02 ± 0.14	1.28 ± 0.14	0.95 ± 0.14
P-AMPK/Total AMPK				
Pretest	1.00 ± 0.12	1.40 ± 0.13	1.00 ± 0.16	1.32 ± 0.18
Posttest	0.93 ± 0.13	1.27 ± 0.11	1.11 ± 0.13	1.40 ± 0.18

* $P < 0.05$ vs pretest; [†] $P < 0.05$ vs before.

Values are means ± SEM. Subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}) while receiving either NO₃⁻ supplementation in the form of beetroot juice (BR, $n = 11$) or a control drink (CON, $n = 11$). In both the pretest and the posttest, a needle biopsy was taken from m. vastus lateralis before and after a 30-min simulated time trial (TT) on a cycling ergometer. HIF1- α , hypoxia inducible factor 1- α ; AMPK, adenosine monophosphate-activated protein kinase. See Methods for more details.

for HIF-1 α mRNA content or for the ratio phospho-AMPK/AMPK total in either the pretest or the posttest. Compared with the pretest, preexercise muscle glycogen contents were increased in the posttest ($P < 0.05$), yet there were no differences between the groups at any time. Net glycogen breakdown during the TT was also similar between the pretest and the posttest in both groups.

Plasma nitrate (Fig. 1)

Baseline plasma NO₃⁻ concentrations were similar between groups and were constant throughout the study in CON. Compared with CON, the administration of BR in the pretest increased plasma [NO₃⁻] ~sixfold ($P < 0.05$), with no further increase in the posttest.

Fig. 1. Effect of beetroot juice on plasma nitrate concentration. Values are means ± SEM for plasma nitrate (μM) before (basal), after 4 days (pretest) and 8 weeks (posttest) of beetroot supplementation (●) or a control drink (○). Between the pretest and the posttest, the subjects were enrolled in a 6-week training program in hypoxia (12.5% F_{O2}). See Methods for more details. * $P < 0.05$ vs basal; [†] $P < 0.05$ vs CON.

Discussion

The hypothesis driving this study was that dietary NO₃⁻ supplementation could enhance the effects of IHT on endurance exercise performance. For oral NO₃⁻, intake stimulates NO production on the one hand and NO conceivably plays a role in adaptation to both endurance training and hypoxia on the other hand (Kingwell et al., 1997; Manukhina et al., 2006; Levett et al., 2011; Limberg et al., 2011). However, against our hypothesis, the data shown in this manuscript indicate that chronic dietary NO₃⁻ supplementation in the form of BR adds neither to the effects of IHT to increase VO_{2max} nor does it enhances power output at the anaerobic threshold or TT performance at sea level.

We enrolled moderately trained healthy volunteers in a 6-week high-intensity endurance training program involving five weekly sessions of cycling training (30 min) at a workload corresponding to 4–6 mmol/L circulating blood lactate. Such training program in hypoxia (~4000 m), compared with similar training in normoxia, was previously demonstrated to stimulate some beneficial physiological adaptations in muscles, yet failed to significantly enhance functional capacity at sea level (Vogt et al., 2001). An identical training program in the current study increased VO_{2max} by 5–9%, and enhanced power output at LT4 and during the 30-min TT by ~15%.

We postulated that daily supplementation of NO₃⁻ in the form of BR could enhance the aforementioned effects of hypoxic training on performance during exercise at sea level. In this regard, it is important to note that we started the BR administration already 4 days before the pretest with the express purpose of eliminating the well-documented acute ergogenic effects of NO₃⁻ supplementation in endurance exercise performance (Hoon et al., 2013). Thus, NO₃⁻ intake (0.07 mmol/kg bw) ~2.5 h before the pretest increased plasma NO₃⁻

concentration ~sixfold, which is in line with earlier observations of Wylie et al. (Wylie et al., 2013). Furthermore, the daily extra NO_3^- intake during the training period did not alter the plasma NO_3^- response to the acute BR bolus in the posttest. Against this background, differences between the pretest and posttest reflect the effect of chronic NO_3^- intake in BR vs CON, which did not receive the daily NO_3^- supplements. However, BR stimulated neither the training-induced increments in $\text{VO}_{2\text{max}}$, nor enhanced the gain in power output at LT4 or during the TT from the pretest to the posttest. In fact, for none of the variables measured in either the TT or the incremental $\text{VO}_{2\text{max}}$ test, we found a significant difference between BR and CON. This clearly indicates that in the conditions of the current study, exogenous NO_3^- supplementation failed to augment the effect of hypoxic training to stimulate functional capacity in endurance exercise at sea level.

Several mechanisms may explain these “negative” observations. First, exercise training per se in healthy individuals readily elevates NO bioavailability via increased NOS enzyme expression and activity (Kingwell et al., 1997; Kingwell, 2000; Manukhina et al., 2006). Accordingly, Kingwell et al. (1997) nicely demonstrated that just 4 weeks of cycling training markedly reduced NO_3^- and NO_2^- consumption by the human forearm, which is consistent with increased NO production through the NOS pathway. Given the elevated capacity for NO production via endogenous pathways due to training, it is reasonable to assume that any additional stimulation of NO production from exogenous NO_3^- may be redundant. Such conclusion is corroborated by the finding that the effect of NO_3^- supplementation to improve O_2 efficiency and performance in endurance exercise is much more explicit in normal healthy volunteers than in highly endurance-trained individuals (Christensen et al., 2013; Hoon et al., 2013).

Second, an obvious way to stimulate training adaptation by NO_3^- administration would be to enhance training intensity by the administration of a high NO_3^- dose (~5–8 mmol bolus) 2–3 h before the start of the training sessions. It is now well established that acute NO_3^- intake can enhance O_2 efficiency during exercise in hypoxia and thereby also improve exercise tolerance (Vanhatalo et al., 2011; Masschelein et al., 2012; Muggeridge et al., 2014). We previously also found lower circulating blood lactate concentration due to NO_3^- intake during submaximal constant-load exercise in hypoxia (Masschelein et al., 2012). Furthermore, it has been well documented that NO_3^- supplementation reduces muscle metabolic perturbation for a given submaximal exercise intensity in hypoxia (Vanhatalo et al., 2011). However, this does not necessarily imply that NO_3^- supplementation fully eliminates hypoxic stress during exercise at (simulated) altitude. Rather, it may permit a more efficient O_2 utilization against the face of a lower degree of hypoxic stress. As a result, higher training workloads may be performed,

which in turn is conceivably associated with recruitment of a higher fraction of fast-twitch motor units that are otherwise “blind” to the hypoxic training stimulus. Therefore, NO_3^- intake might be a useful strategy to enhance training adaptations by facilitating higher training intensities during IHT sessions, indeed. Nevertheless, one should also consider the possibility that NO_3^- , by increasing O_2 efficiency, may reduce the degree of hypoxia occurring in muscle cells during IHT, and thereby inhibits additional training adaptations. The NO_3^- administration protocol used in the current study probably was inadequate to stimulate performance in the training sessions. The total daily NO_3^- dose was distributed over three aliquots per day (~1.7 mmol at breakfast, lunch, dinner), and training sessions were scheduled between 2 and 4 h following either breakfast or lunch. Such dosage protocol results in too low circulating peak plasma NO_2^- levels to enhance O_2 efficiency during exercise (Wylie et al., 2013), and thereby improve exercise tolerance in hypoxia. Unfortunately, we could not measure plasma NO_2^- . Earlier studies have shown that circulating plasma $[\text{NO}_2^-]$ increases between 1 and 15 days of NO_3^- administration at a dose of 5.2 mmol/day (Vanhatalo et al., 2010). However, whether this finding can be extrapolated to even longer periods of oral NO_3^- supplementation remains to be established. In addition, as indicated above, the NO_3^- administration protocol used was successful to chronically increasing plasma NO_3^- concentration, indeed. However, it is difficult to predict, on an individual level, whether this increase in plasma $[\text{NO}_3^-]$ was paralleled by elevated plasma $[\text{NO}_2^-]$, which definitely is the more direct parameter to predict NO availability and biological activity (Dejam et al., 2004).

In the current study during the intervention period, we administered 300–400 mg (0.07 mmol/kg bw) of molecular NO_3^- per day. This intake rate is substantially higher than the mean of ~90 mg daily in an average Western population and within the range of vegetarian diets delivering up to 1500 mg of NO_3^- per day (Hill et al., 1996; Hord et al., 2009; Mitek et al., 2013). Based on this comparison, it is probably reasonable to conclude that increasing daily NO_3^- intake just by intermittent NO_3^- intake via meals, snacks, and drinks is not an adequate strategy to enhance training adaptations in hypoxia. Against the prevailing opinion to date, emerging evidence indicates that diets rich in NO_3^- via fruits and vegetables do not increase the prevalence of stomach cancer. Furthermore, a series of recent studies have shown beneficial effects of dietary NO_3^- intake in cardiovascular and metabolic disease. Still, long-term high-dose NO_3^- supplementation should still be approached with caution till further scientific research on the safety of high-dose NO_3^- boluses typically used by athletes has been done (Bryan et al., 2012; Weitzberg & Lundberg, 2013).

It is well documented that AMPK probably plays an important role in muscular metabolic and structural adaptations to exercise training (Richter & Ruderman,

2009). Furthermore, exercise effects on muscle not only depend on exercise intensity but also on the degree of intramyocellular O_2 desaturation (Geiser et al., 2001; Hoppeler & Vogt, 2001). Therefore, it is believed that activation of HIF-1 is necessary for training adaptation by induction of “hypoxia-sensitive genes” such as vascular endothelial growth factor or glycolytic enzymes, as well as other metabolic proteins (Hoppeler et al., 2008). The 30-min TT per se tended to increase AMPK phosphorylation status both in the pretest and the posttest, yet there was no difference between the experimental groups at any time, either for AMPK total protein content or for the phosphorylated fraction. By analogy, irrespective of the experimental condition, HIF-1 α mRNA contents were similar between the pretest and the posttest both before and after exercise. Thus, consistent with the absence of beneficial effects on functional capacity, NO_3^- intake did not alter the AMPK and HIF-1 α responses to the 6-week training period. Because glycogen breakdown for a given rate of muscle contractions is exaggerated by hypoxia (Parolin et al., 2000), we decided also to assess the effect of the training interventions on net glycogen breakdown during the TT. Training expectedly increased basal muscle glycogen content in both CON and BR, but exercise-induced net glycogen breakdown was similar between groups in both the pretest and the posttest.

In conclusion, the present study clearly demonstrates that long-term low-dose dietary NO_3^- supplementation does not enhance the effects of intermittent endurance training in hypoxia on either the anaerobic threshold, VO_{2max} , or endurance exercise performance at sea level.

Perspectives

The hypothesis driving this study was that daily NO_3^- supplementation could possibly enhance the effects of hypoxic training on sea-level performance. However, one could also argue that suppression of muscular O_2 deficiency during contractions by improved O_2 efficiency due to NO_3^- intake, in fact, might inhibit training adaptation. Yet we found similar training adaptations to hypoxic training, irrespective of whether the training was done alone or in conjunction with daily NO_3^- intake. Still, our data do not exclude that higher daily doses of NO_3^- , probably best to be administered as a bolus 2–3 h prior to the training sessions, or just longer periods of NO_3^- intake, could still affect training adaptations. It is recommended that such long-term studies also address the impact of the NO_3^- administration protocol on primary health parameters.

Key words: Nitric oxide, altitude, high-intensity endurance training, aerobic capacity.

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