Applied Physiology, Nutrition, and Metabolism
Physiologie appliquée, nutrition et métabolisme

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Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2015-0400.R1
Manuscript Type:	Article
Date Submitted by the Author:	30-Sep-2015
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Keyword:	nitrate, neurovascular coupling, cognition, hypoxia

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Running head: Nitrate, neurovascular coupling, and cognition in hypoxia

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Abstract

The matching of oxygen supply to neural demand (i.e. neurovascular coupling [NVC]) is an important determinant of cognitive performance. The impact of hypoxia on NVC remains poorly characterized. NVC is partially modulated by nitric oxide (NO) which may initially decrease in hypoxia. This study investigated the effect of acute NO-donor (nitrate) supplementation on NVC and cognitive function in hypoxia. Twenty healthy men participated in this randomized, double-blind, crossover design study. Following normoxic cognitive/NVC testing, participants consumed either nitrate (NIT) or a NIT-depleted placebo (PLA). Participants then underwent 120 min of hypoxia ($11.6 \pm 0.1\% \text{ O}_2$) and all cognitive/NVC testing was repeated. NVC was assessed as change in middle cerebral artery (MCA) blood flow during a cognitive task (incongruent Stroop) using Transcranial Doppler. Additional computerized cognitive testing assessed separately targeted memory, executive function, attention, sensorimotor and social cognition domains. Salivary nitrite significantly increased following supplementation in hypoxia for NIT ($+2.6 \pm 1.0 \text{ AU}$) compared to PLA ($+0.2 \pm 0.3 \text{ AU}$; $p < 0.05$). Memory performance (-6 ± 13 correct) significantly decreased ($p < 0.05$) in hypoxia while all other cognitive domains were unchanged in hypoxia for both PLA and NIT conditions ($p > 0.05$). MCA flow increased during Stroop similarly in normoxia (PLA $+5 \pm 6$, NIT $+7 \pm 7 \text{ cm} \cdot \text{s}^{-1}$) and hypoxia (PLA $+5 \pm 9$, NIT $+6 \pm 7 \text{ cm} \cdot \text{s}^{-1}$) ($p < 0.05$) and this increase was not altered by PLA or NIT ($p > 0.05$). In conclusion, acute hypoxia resulted in significant reductions in memory concomitant with preservation of executive function, attention, and sensorimotor function. Hypoxia had no effect on NVC. Acute NIT supplementation had no effect on NVC or cognitive performance in hypoxia.

Key words: Nitrate, neurovascular coupling, cognition, hypoxia

Introduction

Normal cerebral function is a critical determinant of cognitive performance and is dependent on oxygen supply delivered by the cerebrovasculature (Chen et al. 2013). As brain activity increases, blood flow must increase to meet neural/metabolic demands and support cognitive activity (Vingerhoets and Stroobant 1999), a process known as neurovascular coupling (NVC) (Iadecola 2004; Attwell et al. 2010). In hypoxic environments, reductions in arterial oxygen saturation are offset by compensatory vasodilation (Casey and Joyner 2011) and subsequent augmented blood flow (Jia et al. 2011; Willie et al. 2014), the degree of which is proportional to the degree of hypoxemia. In the cerebrovasculature, this compensatory hyperemia manifests as increased extracranial (i.e. carotid) (Lewis et al. 2014a; Lewis et al. 2014b) and intracranial (i.e. cerebral) blood flow compared to normoxia (Fan et al. 2010). Blood flow regulation in this setting may be partially controlled by nitric oxide (NO) (Van Mil et al. 2002). NO is released from the vascular endothelium and acts to relax vascular smooth muscle, eliciting vasodilation and increased blood flow (Nichols and O'Rourke 2005). In addition to its central role as a regulator of blood flow (Levett et al. 2011), NO ensures optimal hyperemic response to neural activity (NVC) (Attwell et al. 2010), is necessary for hypoxia-induced cerebral vasodilation (Van Mil et al. 2002), and appears to play an integral role in adaptations to hypoxia (Levett et al. 2011). Upon initial exposure to systemic hypoxia (e.g. acute ascent to high altitude), endothelial dysfunction and reductions in NO production have been noted (Duplain et al. 2000). Consequently, reductions in NO bioavailability with hypoxic exposure may interfere with NVC and thereby impact cognitive performance.

Dietary nitrate supplementation may be a novel means of favorably impacting NVC and cognitive function with acute systemic hypoxia exposure. NO can be synthesized endogenously by NO synthases or from the nitrate-nitrite-NO pathway; dietary nitrate, a natural ingredient of beetroots, vegetables, and leafy greens can be reduced to nitrite and increase NO formation (Hord et al. 2009). Unlike the enzymatic reaction to produce NO, the conversion of nitrite to NO takes place preferentially

under hypoxic conditions (Lundberg et al. 2008). The brain can use nitrate as a direct functional source of NO (Piknova et al. 2011). Increased nitrite concentration via dietary nitrate consumption enhances blood flow at rest (Presley et al. 2011) and increases endothelial function and NO production during neuronal activity, augmenting subsequent NVC during increased cognitive demand (Aamand et al. 2013) which may explain select improvements in cognitive function following acute nitrate supplementation in normoxia (Wightman et al. 2015). Thus, dietary nitrate supplementation in hypoxia might improve NVC and thereby attenuate decrements in hypoxia-sensitive cognitive domains, but this has yet to be explored.

The purpose of this study was to investigate the effects of acute nitrate supplementation on NVC (operationally defined as change in cerebral blood flow from rest to cognitive engagement) and global cognitive function in hypoxia. It was hypothesized that compared to an inert placebo, nitrate supplementation would enhance NVC (manifesting as greater increases in cerebral blood flow measured during cognitive engagement) and improve cognitive function in hypoxia.

Materials and Methods

Participants

Twenty-four recreationally active men in overall good health were recruited from the local University community for this study. Two participants were lost to follow-up and one participant experienced a syncopal episode upon hypoxic exposure and could not complete the study. Additionally, one participant was excluded from data analysis due to excessive time between trials (>4 weeks), leaving 20 participants (23 ± 3 yrs, BMI 24.6 ± 2.8 kg·m⁻², body fat $13.3 \pm 6.8\%$, hemoglobin 14.7 ± 1.5 g/dL) for final analyses. Exclusion criteria included self-reported (determined from a health history questionnaire) smoking, hypertension, diabetes mellitus, hyperlipidemia, pulmonary disease, renal disease, neurological disease, or peripheral artery disease. Hemoglobin concentration was assessed at baseline via finger-stick blood sample and microcuvette (The Hemocue Hemoglobin System, Hb201+;

Angelholm, Sweden) to screen for anemia (defined as Hb < 13.5 g/dL). Participants were not taking any medications at the time of the study and were asked to refrain from dietary supplement use for the duration of the study. This study was approved by the Syracuse University Institutional Review Board and all participants provided written informed consent prior to study initiation. Testing was conducted at the same time of day within-participants in a temperature-controlled laboratory. Participants were instructed to fast for ≥ 3 hours and avoid vigorous exercise and avoid consuming caffeine and alcohol the day of testing. Additionally, participants were given a list of high-nitrate foods to avoid for the 2 days prior to experimental testing.

Design

This study utilized a randomized, double-blind, crossover-design. All participants completed two experimental trials separated by at least 72 hrs. Trials consisted of ingesting either 0.45 g nitrate bolus (Beet It Sports Shot; NIT) or an inert placebo (PLA) prior to hypoxic exposure. On the second experimental visit participants underwent the opposite treatment condition (i.e. if they received NIT on the first visit then they received PLA on the subsequent visit and *vice versa*). Both supplements were identical in appearance, taste, volume (70 mL) and caloric value (4 g protein, 20 g carbohydrate, 0.2 g fat, <0.5 g fiber, <0.1 g sodium; Beet It, James White Ltd, Ipswich), however PLA was depleted of nitrate through a specialized manufacturing process (previously reported nitrate content: placebo ≈ 0.01 mmol vs nitrate-rich ≈ 5.0 mmol (Muggeridge et al. 2013b)). The dose of nitrate used in this study has been previously reported to significantly increase plasma nitrate and nitrite concentration and elicit biological effects in normoxia (Muggeridge et al. 2013a; Wightman et al. 2015), and hypoxia (2,500 m)(Muggeridge et al. 2013b) and is in-line with manufacturer recommendations.

Height and weight was assessed via stadiometer and electronic scale, respectively, and body composition was estimated via air displacement plethysmography (BodPod; COSMED, Concord, CA).

Upon arrival to the laboratory, participants rested in the supine position for 10 minutes before normoxic-baseline vascular and cognitive measures were assessed. Vascular measures were collected in the supine position and in the same order under normoxic and hypoxic conditions. After completion of vascular measures participants underwent salivary nitrite testing as a marker of nitrate metabolism, followed by cognitive testing. Participants then ingested either a) NIT or b) PLA immediately prior to entering the normobaric hypoxic chamber (FiO_2 $11.6 \pm 0.1\%$, $\approx 4,600\text{m}$; Hypoxico Systems, New York, NY). Oxygen concentration was measured using an oxygen monitor (PureAire Monitoring Systems Inc., Lake Zurich, IL) secured inside the hypoxic chamber. Our hypoxic stimulus was chosen based on previous research that established 4000-5000 m as the critical altitude for changes in cognitive function (Babbar and Agarwal 2012). Participants remained in hypoxia for 105 minutes before undergoing hypoxic-vascular and cognitive testing. Our timeline was designed such that cognitive testing would occur concordant with peak nitrate availability (≈ 2 hrs post-nitrate ingestion) as suggested by previous literature (Presley et al. 2011).

Measures

Quantifying the Hypoxic Stimulus

Arterial oxygen saturation was assessed using a reflectance pulse oximeter placed on the forehead (Nonin Medical, Plymouth, MN) in order to quantify the hypoxic stimulus. End-tidal CO_2 (EtCO_2) was measured to ensure CO_2 levels were comparable between treatments (Nellcor OxiMax, Covidien, Mansfield, MA) at normoxic and hypoxic baselines with sampling lines secured directly under the nostrils. Participants rested for 10 minutes prior assessment to ensure stable resting values. Data was collected over a 5-minute period with triplicate measures taken during minutes 2-4 and averaged.

Salivary Nitrite

Salivary nitrite was qualitatively assessed using salivary test strips (Berkeley Test, Berkeley, CA). A salivary absorbent pad was placed under the tongue for 3-5 s and then pressed against a reagent strip. The resulting color was compared to a colored scale to qualitatively assess nitrite availability. Nitrite availability was assessed in 1) normoxia to ensure all participants began with similar, low levels of salivary nitrite; and 2) hypoxia ($\approx$ 2 hours after nitrate ingestion) to document changes in nitrite availability following supplementation.

Neurovascular Coupling

Change in cerebral blood flow measured during cognitive perturbations has been reported to reflect changes in cerebral metabolism (i.e. NVC), and the middle cerebral artery (MCA) is the most commonly interrogated vessel in functional transcranial Doppler studies (Sorond et al. 2013; Bakker et al. 2014; Lupo et al. 2015; Payne et al. 2015; Wolf 2015). This vessel was chosen since the MCA accounts for roughly 80% of total brain blood flow to numerous regions of the brain, including regions essential for executive functions (assessed during our NVC protocol described below). We further assessed common carotid artery (CCA) flow during cognitive perturbation to compliment MCA measures since some regulation of intracranial flow may occur extracranially (Willie et al. 2014). Previous data from our laboratory (unpublished observation) has revealed that there are no significant differences between primary outcome measures across three time points during cognitive perturbation (Stroop task). Thus, CCA blood flow (described below) was assessed once during the Stroop task beginning 30-seconds after task initiation. All NVC metrics were calculated as absolute change from baseline.

In this study we measured NVC during a computerized, modified incongruent Stroop color-word interference task (E-Prime, Psychology Software Tools Inc, Sharpsburg, PA). This test has been previously used as a mental stressor and means of assessing NVC in our laboratory (Heffernan et al. 2014). All participants were familiarized with the Stroop task prior to experimental testing in order to

control for learning effects. The Stroop task was completed in the supine position with the head tilted slightly back, thereby optimizing the imaging window of the carotid artery. The viewing display for normoxic testing was a specialized wall-mounted 107-cm flatscreen television that extended over the participant. Font was displayed approximately 102-cm above the participant with 3.0-cm font on a black background. For hypoxic testing, the Stroop task was projected onto the ceiling of the chamber using a computer-interfaced projector (IN1100, InFocus, Portland, OR) that displayed the task approximately 160-cm above the participant with 4.5-cm font. Despite the task being displayed farther away from the participant in hypoxia, the ratio between viewing distance (cm) and font size (cm) was comparable to normoxia (34:1 normoxia, 36:1 hypoxia).

The NVC protocol began when participants were presented with a white crosshair in the center of the viewing window for approximately 3-seconds. A target word was displayed in incongruous colors (e.g. the word “blue” written in the color red), with four names of response colors presented similarly (e.g. the word “red” written in the color blue). The task was to use a response clicker to identify the color that matched the target word displayed as quickly as possible. The response colors (1-4) corresponded to the remote clicker buttons (1-4) which the participant manipulated using the digits on their dominant side (index finger – pinky finger). This task lasted 4-minutes in duration, which has been previously been shown to elicit changes in heart rate and blood pressure (Heffernan et al. 2014).

Participant’s identification accuracy was titrated to 60% in order to produce equivalent hemodynamic responses across sea level and HA Stroop testing. This was achieved via manipulation of the inter-item timing intervals (ITI; i.e. the time between presentation of each color-word challenge). For every three consecutive items answered correctly the ITI was decreased by 300 ms (shortest ITI of 400 ms). Similarly, three consecutive missed items would increase the ITI by 300 ms (longest ITI of 5,000

195 ms). If the participant did not respond in time, a large “TOO LATE!” prompt was displayed before the
196 next item was displayed.

197 *Cerebral Blood Flow*

198 Middle cerebral artery (MCA) blood flow velocity was assessed using a 2-MHz transcranial
199 Doppler (TCD) probe (DWL Doppler Box-X, Compumedics, Germany) applied to the left temporal
200 window. Mean blood flow velocity (MnV) was measured at a depth of 50-65mm, as is commonly
201 reported for MCA measurements (Xu et al. 2012). All repeated measurements within each participant
202 were taken at the same depth and position to ensure recapture of the same cerebral artery. The
203 envelope of the velocity spectrum and mean velocity was calculated by a standard algorithm
204 implemented on the instrument with use of a fast Fourier transform. MCA blood flow velocity was also
205 expressed relative to blood pressure by calculating MCA conductance as $(\text{MCA MnV}/\text{MAP}) \times 100$, where
206 MAP is mean arterial pressure calculated as $1/3 \text{ systolic} + 2/3 \text{ diastolic blood pressure}$.

207 *Common Carotid Artery Blood Flow*

208 Images of the distal left common carotid artery (CCA) were obtained using Doppler ultrasound
209 (ProSound $\alpha 7$, Aloka, Tokyo, Japan) and a 7.5-10.0 MHz linear-array probe. CCA diameters were
210 measured from inside the near-wall intima-media to far-wall intima-media across a 5 mm region of
211 interest via semi-automated digital calipers during systole and diastole (indicated by the R-wave and end
212 of the T-wave from simultaneous ECG gating). Average diameter was calculated as $(1/3 \text{ systolic}$
213 $\text{diameter} + 2/3 \text{ diastolic diameter})$. Mean blood flow velocities (MnV) were measured using Doppler-
214 ultrasound with an insonation angle $\leq 60^\circ$ for all measures and sample volume manually adjusted to
215 encompass the entire vessel. Mean velocity was calculated as: $\text{MnV} = \int V(t) dt / \text{FT}$, where $\int V(t) dt$ is the
216 velocity-time integral of the velocity waveform and FT is flow time. Velocity waveforms were measured.

Blood flow was calculated as $\pi \times (1/3 \text{ systolic radius} + 2/3 \text{ diastolic radius})^2 \times \text{MnV} \times 60$. All images were stored for later offline analysis by a single investigator who remained blinded regarding which treatment contained nitrate.

Brachial Blood Pressure

Systolic blood pressure (SBP) and diastolic brachial blood pressure (DBP) were measured prior to each set of vascular measures (at normoxia and hypoxia) using a validated, automated oscillometric cuff (EW3109, Panasonic Electric Works, Secaucus NJ). Pressures were taken in duplicate and averaged. If values were different by more than 5 mmHg a third measure was obtained and the average of the 2 closest measures was used for subsequent analyses.

Computer-Based Cognitive Assessment

Global assessment of cognitive function was made through a validated computer-based program (WebNeuro; Brain Resource, San Francisco, CA) which has sound test-retest reliability (Williams et al. 2005). Participants engaged in a series of cognitive tasks while sitting at a laptop computer with a standard mouse/keyboard. The cognitive tests covered sensorimotor, memory, social cognition, attention, and executive function domains and the specific tasks have been described in detail elsewhere (Silverstein et al. 2007). Brief summaries of the tests are provided in Table 1.

Statistical Analyses

Effect size values were estimated from previous literature for cerebral blood flow (0.65)(Poulin and Robbins 1996), reaction time (0.48)(Li et al. 2012) and memory (0.89)(Wang et al. 2013) in hypoxia, identifying an average effect size of 0.67. Therefore, for a power of 0.80 with alpha set as 0.05 for a two-tailed t-test, approximately 20 participants was determined to be sufficient to observe similar changes in blood flow and cognitive function during hypoxia.

All data are presented as mean $\pm$ standard deviation. All data was normally distributed, as tested using histograms, Q-Q plots, and Shapiro-Wilk tests, thus no variables were logarithmically transformed. The effect of altitude exposure was tested using paired t-tests between normoxia and hypoxia. The effect of NIT was tested using paired t-tests between NIT and PLA in hypoxia. NVC was assessed using absolute Δ scores, calculated as cognitive engagement values – baseline values, for each treatment (PLA, NIT) and condition (normoxia, hypoxia). A one-sample t-test compared NVC change scores to zero to determine if there was significant hemodynamic coupling during cognitive activity. Significance was set *a priori* at $p < 0.05$.

Results

The duration of hypoxic exposure (165 ± 8 min, PLA; 161 ± 8 min, NIT; $p = 0.076$) and percent oxygen in the hypoxic chamber ($11.6 \pm 0.1\%$, PLA; $11.7 \pm 0.1\%$, NIT; $p = 0.34$) were not significantly different between treatments. Hypoxia resulted in similar significant decreases in SaO_2 (98 ± 2 vs $75 \pm 6\%$ PLA; 98 ± 2 vs $75 \pm 7\%$ NIT; $p < 0.001$) and ET- CO_2 (39 ± 2 vs 33 ± 2 mmHg PLA; 39 ± 3 vs 34 ± 2 mmHg NIT; $p < 0.001$) in both treatments compared to normoxia. Salivary nitrite was similar between treatments under normoxic conditions (0.1 ± 0.2 AU PLA, 0.1 ± 0.2 AU NIT; $p = 0.49$) prior to NIT/PLA consumption. Following acute supplementation, salivary nitrite was significantly greater in hypoxia for NIT (2.7 ± 1.0 AU) compared to PLA (0.2 ± 0.3 AU; $p < 0.001$).

Effect of hypoxia on cerebrovascular function

CCA diameter increased with acute exposure to hypoxia ($p < 0.05$). The increase in CCA diameter was similar with PLA and NIT treatments ($p > 0.05$). MCA mean flow velocity and CCA blood flow increased at rest in hypoxia for both PLA and NIT treatments ($p < 0.05$; Table 2). Brachial MAP was similar in both treatments under hypoxia and normoxia ($p > 0.05$). MCA conductance tended to increase in hypoxia compared to normoxia although it did not reach statistical significant ($p = 0.13$). Overall,

there was no differences in cerebrovascular measures between PLA and NIT under hypoxic exposure ($p > 0.05$), indicating NIT supplementation did not alter resting vascular responses to hypoxia.

Effect of hypoxia on neurovascular coupling

There were increases in MCA mean flow velocity during Stroop task compared to baseline in both treatments and conditions, providing evidence of NVC ($p < 0.05$; Table 3). For both PLA and NIT treatments, increase in MCA flow velocity during Stroop (ΔmV ; Table 2) was not different in hypoxia compared to normoxia ($p > 0.05$). MCA conductance was unaltered during cognitive activity in both hypoxia and normoxia ($p > 0.05$). CCA diameter increased during Stroop compared to resting baseline in normoxia ($p < 0.01$). CCA diameter change during Stroop was attenuated in both PLA and NIT treatments in hypoxia compared to normoxia ($p < 0.05$). Despite reductions in CCA vasodilation, blood flow during Stroop was ultimately similar between hypoxia and normoxia within both treatments ($p < 0.05$) which may have resulted from a tendency for attenuated reductions in CCA mean velocity during Stroop in hypoxia ($p = 0.062$). Overall, there were no differences in cerebrovascular response to Stroop in hypoxia between NIT and PLA treatments ($p > 0.05$), indicating NIT did not significantly alter NVC under hypoxic conditions compared to PLA.

Effect of hypoxia on cognitive function

There were no differences in cognitive function at normoxic baseline between PLA and NIT treatments ($p > 0.05$). A significant effect of hypoxia was detected within the memory and information processing domains (Table 4). Memory recognition was lower in hypoxia compared to normoxia in both PLA and NIT treatments ($p < 0.05$). This was driven by greater intrusion errors and lower memory recognition performance in both PLA and NIT treatments in hypoxia ($p < 0.05$). Accuracy and RT during the visual interference task was improved in both PLA and NIT treatments in hypoxia compared to normoxia ($p < 0.05$). Emotion recognition index was lower in both PLA and NIT treatments in hypoxia compared to

normoxia ($p < 0.05$). Cognitive performance in the remaining cognitive domains were not affected by hypoxic exposure in either PLA or NIT treatments ($p > 0.05$). The reductions in aforementioned domains of cognitive function in hypoxia were similar between PLA and NIT treatments ($p > 0.05$ for interaction), indicating NIT supplementation was not effective in altering cognitive function during hypoxic exposure. There were no changes in any other cognitive domain from normoxia to hypoxia with either PLA or NIT ($p > 0.05$).

Discussion

This study investigated the effect of acute dietary nitrate supplementation in the form of beetroot juice on NVC and cognitive function under hypoxic conditions. The overarching hypothesis was that nitrate supplementation would result in greater NO formation and act to increase vasodilation, blood flow, and oxygen delivery to the cerebrovasculature, thereby optimizing NVC and improving cognitive performance following rapid exposure to systemic hypoxia. The primary findings of the study were as follows: 1) NVC (assessed as the increase in MCA mean flow velocity during cognitive engagement) was similar in normoxia versus hypoxia; 2) while aspects of memory performance were reduced during hypoxia, other aspects of cognitive performance (executive function, attention, sensorimotor processing) were preserved; 3) nitrate did not alter cognitive performance nor did it alter cerebral blood flow during cognitive engagement (NVC) in hypoxia. Thus, acute dietary nitrate supplementation does not affect NVC or cognitive performance under hypoxic conditions in young, healthy men.

Acute exposure to systemic hypoxia results in complex alterations in neurophysiological and psychological function. Examining the impact of nitrate supplementation on NVC and cognitive function during hypoxia has important implications for populations regularly exposed to hypoxic conditions such as mountaineers (Merz et al. 2013), aircraft pilots (Petrassi et al. 2012), and military personnel (Adam

et al. 2008)), and to clinical populations that suffer acute ischemic events (Anderson and Arciniegas 2010; Peskine et al. 2010). Under normoxic conditions, short-term nitrate loading (1-3 days) has been reported to increase regional cerebral perfusion in older adults (Presley et al. 2011), and reduce hyperemic lag times during visual stimulation (Aamand et al. 2013), indicative of improved NVC. Additionally, normoxic data would suggest that nitrate supplementation can increase brain perfusion (Presley et al. 2011), although this is not a universal finding (Aamand et al. 2013), and acutely improve cognitive function during a mental fatigue protocol (Wightman et al. 2015). With this information as our foundation, we set out to explore the effect of acute nitrate supplementation on NVC and cognitive function in systemic hypoxia.

Oxygen tension is a fundamental factor in determining blood flow to target organs such as skeletal muscle or the brain. Hypoxic conditions result in a compensatory hyperemic response (Jia et al. 2011) in order to offset reductions in arterial oxygen content. We noted significant increases in CCA diameter, CCA flow and MCA flow velocity with hypoxia. Nearly 80% of CCA blood flow feeds the internal carotid, which evolves into the MCA and provides approximately 80% of the blood supply to the brain (Poulin and Robbins 1996; Farkas and Luiten 2001). Thus some intracranial flow regulation may occur extracranially (Willie et al. 2014). Our findings are consistent with recent comprehensive studies by Lewis et al. (2014) who reported significant increases in CCA dilation following slightly more prolonged exposure (72-96 hr) after both normobaric (Lewis et al. 2014b) and hypobaric hypoxia (Lewis et al. 2014a). Moreover, NVC was largely maintained during hypoxia in the current study. Thus, there were additional increases in carotid and cerebral flow during cognitive engagement above levels initially caused by hypoxia. Maintenance of NVC occurred despite an attenuated CCA dilatory response during cognitive engagement in hypoxia. Total inflow volume is ultimately determined by changes in vessel diameter and flow velocity. It is possible that the CCA was maximally dilated under the current hypoxic stimuli, resulting in cognitive activity-dependent hyperemia achieved through alteration of blood flow

velocity rather than diameter, although future studies should directly interrogate this observation using endothelium-independent dilators (i.e. sodium nitroprusside).

The current study observed significant decreases in memory function following approximately 2.5 hours of hypoxia. Specifically, there were reductions in verbal memory and intrusion indices, resulting from decrements in immediate and delayed memory accuracy and greater error rates, respectively. These decrements in the memory domain are consistent with previous reports (Dykiert et al. 2010; Li et al. 2012; Malle et al. 2013; Wang et al. 2013) across a variety of hypoxic stimuli ranging from 2,800m (Wu et al. 2002) to 9,449m (Malle et al. 2013). There was also significant dysfunction with regards to emotion recognition. Emotion recognition in this study was assessed as 1) the ability to correctly identify emotion based on facial expressions and 2) the ability to recall ($\approx$ 10 minutes later) which facial expressions had been previously presented. Thus, it is possible that the reductions in emotion recognition observed herein may be partially related to/driven by impaired memory function that potentially impacted the emotion recall performance. We noted no significant changes for the remaining cognitive domains. Indeed, some domains of cognitive function may not be affected by hypoxia. Performance on simple tasks, such as 2-choice RT and finger tapping, is maintained at altitudes below 6,000m (Virues-Ortega et al. 2004; Petrassi et al. 2012). Consistent with these reports, we observed no significant changes in finger tapping speed, choice RT, or go-no-go tasks. We also observed no effect of hypoxia on verbal learning rate and executive function and this too is in line with previous reports (Paul and Fraser 1994; Asmaro et al. 2013). This supports a recent review (Petrassi et al. 2012) suggesting that some domains may not be sensitive to acute hypoxia (Pavlicek et al. 2005; Petrassi et al. 2012), or perhaps require longer exposure or more extreme hypoxia to observe effects.

Acute nitrate supplementation had no effect on the hypoxic vascular response, NVC or cognitive function. The dose of nitrate used in this study has been reported to increase plasma levels of nitrite

and nitrate (Muggeridge et al. 2013b) and affect cognitive function in normoxia (Wightman et al. 2015), although this positive effect on cognition is not a universal finding (Kelly et al. 2013; Thompson et al. 2014). Further increasing the dose in order to maximize effects may not be efficacious since nitrate loading (≈ 50 mmol/day) does not increase cerebral oxygenation in hypoxia (Masschelein et al. 2012) and supra-physiological concentrations of nitrite have been shown to impair NVC by increasing local resting cerebral flow, thus limiting cerebrovascular reserve in response to neuronal activation (Piknova et al. 2011). Nitrite supplementation has been shown to increase NVC in animal models, but only after inhibition of normal NO production within the brain (Piknova et al. 2011). Thus, nitrate supplementation in our study may not have altered hypoxic vasodilation and NVC because our young participants did not have impaired NO-bioavailability under hypoxic conditions. Ultimately, the mechanisms responsible for hypoxia-induced cerebrovascular vasodilation and hyperemia may be multifactorial and rely on multiple, redundant pathways similar to those observed with the peripheral vasodilatory responses to exercise (Joyner and Casey 2009). A recent review has suggested these cerebral mechanisms may not only involve NO, but also several other factors such as adenosine, prostaglandins, PaO_2 and anaerobic neuronal metabolism (Willie et al. 2014). Moreover, there may also be competing neural mechanisms related to regional release of neurotransmitters/neuropeptides (i.e. brain-derived neurotrophic factor, vasopressin, neuropeptide Y, dehydroepiandrosterone etc) along with disparate changes in oxygen extraction at the tissue level.

Preservation of NVC in hypoxia may thusly preserve a majority of cognitive functions as seen herein. The question then arises: why were some cognitive functions conserved while others detrimentally impacted? Domains related to executive function, reaction time, attention, concentration, and information processing (arguably considered more vital for survival in a fight-or-flight situation) are largely under prefrontal cortex regulation while domains such as verbal memory and emotion recognition memory may be of hippocampal origin. The hippocampus has been found to be

more sensitive to hypoxia-mediated oxidative stress and subsequent hypoxic-ischemic injury than the cortex (Maiti et al. 2006; Hota et al. 2007; Maiti et al. 2008), which has been linked to heterogeneity in regional hemodynamic responsiveness to hypoxia (Dunn et al. 1999). Moreover, these hypoxic alterations in hemodynamics and oxidative stress in the hippocampus have been specifically linked to impairments in memory (Maiti et al. 2008). Future studies should explore both anterior cerebral arteries (frontal cortex) and posterior cerebral arteries (hippocampus) as part of NVC protocols that explore cognitive function in hypoxia.

Limitations and future directions

The participants in the current study were generally young (23 ± 3 yrs), healthy males which limits applicability to other populations. Results may differ if this study was conducted in females based on hormonal differences introduced by the menstrual cycle and the subsequent timing of measures during the cycle. Investigating the effects of hypoxia on the cognitive and cerebrovascular function is important across a large range of ages because vascular responses to hypoxia may change with age (Petrassi et al. 2012). Older adults or those with multiple cardiovascular risk factors have been reported to experience neurovascular uncoupling (D'Esposito et al. 1999; Stroobant and Vingerhoets 2000; Groschel et al. 2007) and suffer from impaired sympatholysis (Dinenno et al. 2005; Casey et al. 2014) which is of particular importance since a portion of hypoxic hyperemia is due to sympatholysis overriding sympathetic-mediated constriction (Dinenno et al. 2003). Studying the acute effect of nitrate ingestion on NVC in hypoxia in this population may be beneficial as nitrate has been shown to restore sympatholysis in older adults during hypoxic exercise (Casey et al. 2014) and alter NVC in older adults (Presley et al. 2011). It is possible that individuals repeatedly exposed to subacute hypoxia/ischemia may respond differently to nitrate supplementation due to hypoxic preconditioning. Studies suggest that repeated subacute hypoxic exposure may offer neurovascular protection (Poinsatte et al. 2015),

stimulate cerebrovascular remodeling (Boroujerdi and Milner 2015), and may improve oxygen saturation (Foster et al. 2014) and abrogate oxidative stress (Berger et al. 2015) during additional ischemic events. Whether hypoxic preconditioning alters the effect of nitrate on NVC and cognitive function in hypoxia was beyond the scope of the current study and requires future research.

Cerebral blood flow is dependent on arterial gases, blood pressure, and neural activity (Willie et al. 2014). Hypoxia is accompanied by a hyperventilatory response that, although intended to defend against arterial hypoxemia, may have a profound effect on the cerebrovasculature via hyperventilation-induced hypocapnic vasoconstriction (Brugniaux et al. 2007). In the current study, participants were exposed to similar hypoxic stimuli (severity and duration), and demonstrated similar acute hyperventilation responses to hypoxia (evident by similar reductions in ET-CO_2), thus we do not believe the differences in ventilatory responses to hypoxia altered the cerebrovascular responses between treatments. Mean arterial pressure was not significantly different between conditions or treatments, however autoregulation may be compromised in hypoxia (Nishimura et al. 2010) although this is somewhat debated (Querido et al. 2013). Cerebral perfusion becomes more dependent on pressure in the presence of impaired cerebral autoregulation. Thus, small changes in pressure may influence NVC. Indeed, when cerebral blood flow velocity was expressed as conductance (i.e flow relative to pressure) we noted no significant NVC, suggesting that the increases in MCA mean velocity during cognitive activity may be the result of a combination of blood pressure-mediated effects of mental stress and neural activity. Future research is required to further tease out the independent and combined roles of CO_2 , blood pressure/autoregulation, and neural activity on cerebral hemodynamics under hypoxic conditions.

Conclusions

Our novel assessment of NVC revealed a preservation of carotid and cerebral flow during cognitive engagement in hypoxia that may be related to preferential conservation of select cognitive domains (executive function, reaction time, attention and concentration, information processing) but not others (memory and emotion and recognition). Ultimately, we found that compared to placebo, acute nitrate supplementation did not affect NVC during hypoxic exposure nor did it alter cognitive performance in young healthy men.

Acknowledgments

The authors have no conflicts of interest to disclose. This research was supported by an ACSM Foundation Research Grant from the American College of Sports Medicine Foundation.

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Table 1: Computerized cognitive battery task description.

Domain	Task	Description	Construct
Memory	Memory Recognition	Presented with 20 words during 4 memorization trials. Then presented with 1 target word and 2 distracter words, must correctly identify word from memorization lists. ~10 minutes later, memory recognition trial was repeated	Immediate, Delayed-Recognition Immediate, Delayed Intrusions
Attention	Continuous Performance Test	Presented with series of letters, must respond when the same letter is presented twice in a row	RT Errors
	Forward Digit Span	Presented with series of digits, must correctly enter digits in the order they were presented. Number of digits increased sequentially from 3-7	Recall Span Trials Correct
Executive Function	Switching of Attention	Similar to Trials B task. Presented with series of letters and numbers, must click on numbers and letters in alternating ascending order (1, A, 2, B etc.) as fast as possible	Duration RT Accuracy (%)
	Visual Interference	Presented with target word (e.g. "red"), must identify what color the word says while ignoring the color of the paint as fast as possible.	Correct trials Errors RT
	Verbal Interference	Presented with target word (e.g. "red"), must identify what color the word is painted while ignoring the color the word says as fast as possible.	Correct trials Errors RT
	Maze	Presented with 8x8 grid, must identify/learn the hidden path through the grid, must complete twice without error	Trials Completed Path Learning Time Accuracy (%)
	Go/No-Go	Must respond as quickly as possible when presented with "go" stimuli, and must not respond when presented with "no-go" stimuli	RT Errors
Sensori-motor	Motor Tapping	Tap as fast as possible (30 sec)	Tapping Speed
	Choice RT	Respond to 1 of 2 highlighted circle as fast as possible	Choice RT
Social Cognition	Emotion Recognition	Presented with faces of diverse emotional expression, must correctly identify emotion out of 6 emotional words as quickly as possible. ~ 10 minutes later, had to identify previously viewed faces when presented next to distracter face as fast as possible	Correct (%) RT

RT, Reaction Time

Table 2: Resting cerebrovascular measures across treatments and conditions (mean ± SD)

	Placebo		Nitrate	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Brachial MAP (mmHg)	83 ± 7	87 ± 6	84 ± 6	85 ± 9
CCA Mean diameter (mm)	5.91 ± 0.46	6.60 ± 0.47*	5.75 ± 0.48	6.54 ± 0.45*
CCA Mean velocity (c·ms ⁻¹)	37.2 ± 4.6	37.7 ± 5.5	38.0 ± 3.9	36.7 ± 6.5
CCA Blood flow (ml·s ⁻¹)	621.0 ± 89.9	786.5 ± 139.7*	602.0 ± 88.1	746.5 ± 126.3*
MCA Mean velocity (m·s ⁻¹)	66 ± 15	74 ± 20*	68 ± 16	75 ± 18*
MCA Conductance (cm·s ⁻¹ ·mmHg ⁻¹)	80 ± 19	87 ± 26	82 ± 19	90 ± 22
Heart rate (b·min ⁻¹)	56 ± 9	64 ± 8*	54 ± 7	64 ± 11*
SaO ₂ (%)	98 ± 2	75 ± 6*	98 ± 2	75 ± 7*

MAP, mean arterial pressure; CCA, common carotid artery; MCA, middle cerebral artery; SaO₂, arterial oxygen saturation.

*p < 0.05 vs normoxia

Table 3: Cardiovascular and cerebrovascular change values (cognitive engagement – rest) across treatments and conditions (mean $\pm$ SD)

	Placebo		Nitrate	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Brachial MAP (mmHg)	9 $\pm$ 6*	5 $\pm$ 6*	4 $\pm$ 4*	5 $\pm$ 8*
CCA Mean diameter (mm)	0.16 $\pm$ 0.15*	0.00 $\pm$ 0.20†	0.17 $\pm$ 0.19*	0.07 $\pm$ 0.26†
CCA Mean velocity (cm·s ⁻¹)	- 0.8 $\pm$ 3.1	- 0.4 $\pm$ 3.6^	- 2.0 $\pm$ 3.6*	0.5 $\pm$ 3.1^
CCA Blood flow (ml·s ⁻¹)	16.3 $\pm$ 66.7	- 10.9 $\pm$ 91.6	- 0.8 $\pm$ 67.6	28.3 $\pm$ 90.2
MCA Mean velocity (m·s ⁻¹)	5 $\pm$ 6*	5 $\pm$ 9*	7 $\pm$ 7*	6 $\pm$ 7*
MCA Conductance (cm·s ⁻¹ ·mmHg ⁻¹)	-2 $\pm$ 8	+ 1 $\pm$ 10	-4 $\pm$ 10	+ 2 $\pm$ 12
Heart rate (b·min ⁻¹)	8 $\pm$ 11*	7 $\pm$ 8*	6 $\pm$ 8*	5 $\pm$ 7*
SaO ₂ (%)	0 $\pm$ 2	0 $\pm$ 5	0 $\pm$ 2	1 $\pm$ 4

MAP, mean arterial pressure; CCA, common carotid artery; MCA, middle cerebral artery; SaO₂, arterial oxygen saturation.

*P < 0.05 vs zero; †p < 0.05 vs normoxia; ^trend p = 0.06 vs normoxia

Table 4: Cognitive performance and reaction times by cognitive domain across treatment and condition (mean ± SD)

Domain	Task	Construct	Placebo		Nitrate	
			Normoxia	Hypoxia	Normoxia	Hypoxia
Memory	Memory Recognition	Learning Rate	0 ± 1	0 ± 1	0 ± 1	0 ± 1
		Verbal Memory Index	75 ± 9	70 ± 10*	75 ± 5	71 ± 10*
		Verbal Intrusion Index	5 ± 9	10 ± 10*	5 ± 5	9 ± 10*
Emotion Identification	Emotion Recognition	Emotion Recognition Index	175 ± 9	173 ± 11*	176 ± 6	170 ± 11*
		Average RT (ms)	1671 ± 318	1591 ± 518	1620 ± 281	1520 ± 256
Working Memory Capacity	Digit Span (Forward)	Recall Span (forwards)	7 ± 1	7 ± 2	7 ± 1	6 ± 1
		Trials Correct (forwards)	9 ± 2	9 ± 3^	9 ± 2	7 ± 3^
Information Processing Efficiency	Visu-I (Word)	# Correct w/ Vis-I	17 ± 5	20 ± 4*	17 ± 5	19 ± 5*
		RT w/ Vis-I (ms)	1186 ± 321	969 ± 156*	1108 ± 214	1056 ± 306*
	Verb-I (Color)	# Correct w/ Ver-I	17 ± 4	17 ± 3	18 ± 5	17 ± 5
		RT w/ Ver-I (ms)	1156 ± 278	1146 ± 212	1139 ± 285	1124 ± 208
	Switching of Attention	Duration (ms)	38783 ± 6742	35235 ± 6463	38765 ± 7160	40163 ± 11671
		Connection Time (ms)	1508 ± 285	1382 ± 258	1483 ± 274	1580 ± 455
		Accuracy	0 ± 1	1 ± 1	1 ± 2	1 ± 1
	Choice RT	Choice RT (ms)	343 ± 74	347 ± 32	329 ± 35	351 ± 27
Response Speed	Motor Tapping	Tapping Speed	200 ± 25	199 ± 23	206 ± 23	197 ± 22
Executive Function	Maze	Trials Completed	7 ± 3	8 ± 3	8 ± 2	8 ± 2
		Path Learning Time (ms)	94322 ± 38855	94159 ± 53061	88668 ± 30494	97255 ± 38098
		Accuracy	30 ± 14	28 ± 11	29 ± 12	31 ± 10
Attention and Concentration	Continuous Performance Test	RT (ms)	560 ± 200	519 ± 128	467 ± 69	539 ± 186
		Errors	3 ± 6	2 ± 3	2 ± 2	4 ± 7
Impulsivity	Go/No-Go	Speed (ms)	292 ± 45	283 ± 61	288 ± 44	275 ± 44
		Errors	1 ± 2	1 ± 2	1 ± 3	2 ± 3

RT, reaction time; Visu-I, visual intereference; Verb-I, verbal interference

* p < 0.05 vs normoxia; ^ trend, p < 0.10 vs normoxia