Carriker, CR, Gibson, A, & Mermier, C. The role of the nitrate-nitrite-nitric oxide pathway during hypoxia. J Sport Human Perf 2013;1(4):63-78. doi: 10.12922/jshp.0027.2013

JSHP
JOURNAL OF SPORT AND HUMAN PERFORMANCE

REVIEW OPEN ACCESS

THE ROLE OF THE NITRATE-NITRITE-NITRIC OXIDE PATHWAY DURING HYPOXIA

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ABSTRACT

Recently, dietary nitrate has been shown to reduce oxygen cost during submaximal sea level exercise. The conversion of nitrate to nitrite to nitric oxide has been implicated as an oxygen independent pathway, and as such, may be a potent ergogenic aid during hypoxic conditions as occurs during high altitude exposure. Several studies have noted improved vascular and myocardial function/outcomes when NO was activated prior to ischemia or reperfusion injury. Therefore, increased NO formation/availability during incidence of hypoxia or otherwise impaired blood flow may reduce neuronal damage, improve cognition, and further improve motor function in individuals experiencing ischemic conditions. Consuming dietary nitrate may result in increased NO formation as nitrate consumption has previously been shown to increase plasma nitrite (an in vivo marker of NO production). Increased NO formation during incidence of hypoxia may negate some of the negative consequences of the reduced oxygen diffusion gradient between blood and tissues.

Keywords: Nitrate Supplementation; Oxygen Cost; High Altitude; Performance

INTRODUCTION

Human movement and cognition rely on cerebral processing via an intricate neuronal network. The physiology of cerebral energy metabolism has been recently debated regarding an astrocyte-neuron lactate shuttle or conventional glucose oxidation as viable substrates (1,2). Reduced cerebral oxygenation may occur in ischemic stroke patients or during environmental exposure to high altitude. Cerebral ischemia, under severe cases, may result in not only impaired cognitive and motor function but neuronal damage or degeneration including either neuronal apoptosis or necrosis (3). However, if blood flow to regions of the brain is increased, greater oxygen flux occurs, which may ameliorate the consequences of cerebral ischemia outlined above (4,5). Recently, dietary nitrate has been shown to reduce oxygen cost during submaximal sea level exercise (6). Further, the conversion of nitrate to nitrite to nitric oxide has been implicated as an oxygen independent pathway (7), and as such, may be a potent ergogenic aid during hypoxic conditions as occurs during high altitude exposure.

Initially, nitric oxide (NO) was deemed a potent vasodilator capable of increasing blood flow via vascular smooth muscle relaxation and cyclic GMP accumulation (8). However, NO has also been implicated in a number of other functions such as improved or increased exercise induced glucose uptake in skeletal muscle (9), neurotransmission (10), immune response (11), regulation of mitochondrial respiration (12–14), and glycolysis as mediated by AMPK (15,16). Clinically, NO may ameliorate some of the negative consequences of ischemia/reperfusion injury (reduced infarct size and endothelial dysfunction), especially in the heart (17) and brain (5) . Several studies have noted improved vascular and myocardial function/outcomes when NO was activated prior to ischemia or reperfusion injury (18–20). Therefore, increased NO formation/availability during incidence of hypoxia or otherwise impaired blood flow may reduce neuronal damage, improve cognition, and further improve motor function in individuals experiencing ischemic conditions.

Brain blood flow during exercise

In addition to pathology surrounding stroke or other vascular compromise, as exercise intensity nears maximum, the onset of total body exhaustion is initiated. Such a phenomenon is often commonly recognized as an 'I need to stop' feeling. During such maximal exercise, a slight decline in cardiac output may precede fatigue (21) with a concomitant reduction in brain blood flow (22) while oxygen extraction may be enhanced in cerebral tissue (23). Under such maximal exercise conditions, cerebral oxygen demand remains high and in some instances, oxygen supply may be outpaced by cerebral demand. When this occurs, to avoid damage or other catastrophic failure, motor unit recruitment is reduced (22). A meta-analysis examining cardiovascular training status found that untrained participants had reduced oxygen delivery to the frontal cortex which was determined to be insufficient for demand during high intensity exercise. As a result, untrained participants incurred insufficient cerebral oxygenation when compared to trained counterparts (22).

Over a wide range of exercise intensities from rest through high intensity, global cerebral oxygenation follows a quadratic trend (22). Therefore, cerebral oxygenation increases during low to moderate intensity exercise and begins to level off when approaching high intensity exercise. As exercise intensities reach maximum, cerebral oxygenation falls (22). During actual and imagined exercise, an increase in perceived exertion may initiate an increase in regional cerebral blood flow (within the thalamic region, insular cortex and anterior cingulate cortex or the medial prefrontal region). Participants who experienced an increase in perceived exertion during both actual and imagined exercise also incurred an elevated heart rate and blood pressure (24).

Additionally, when effort/activity is imagined, insular cortex activation increases when a cardiovascular response occurs simultaneously (25). Homeostatic feedback is afferently relayed to the dorsal posterior insula, while the appropriate sensation is manufactured within the anterior insula based on said afferent feedback (26). Given the quadratic trend for cerebral oxygenation mentioned above, during maximal exercise (when the sensation to cease exercise occurs) feedback pertaining to cerebral blood flow/oxygenation may initiate protective signaling mechanisms to down-regulate or stop the exercise/activity prior to catastrophic failure, organ ischemia or injury.

During maximal intensity exercise, the content of deoxygenated hemoglobin in the brain rises while cerebral oxygenation in the prefrontal cortex declines (27). The prefrontal

cortex relays information to the motor cortex, and in the presence of decreased prefrontal oxygenation results in decreased muscle function (28). Under such a relay mechanism, cerebral oxygen desaturation precedes voluntary exhaustion (29). Ultimately, if brain blood flow were increased, perception of effort may be reduced and, during maximal exercise, performance may be improved as motor unit recruitment could remain high for extended durations prior to initiating volitional cessation.

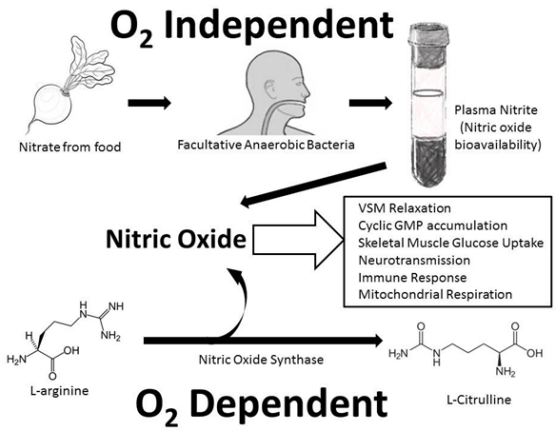

Figure 1. Pathways producing nitric oxide via an oxygen independent (top branch) or oxygen dependent (bottom branch) mechanism. Dietary nitrate is reduced to nitrite via facultative anaerobic bacteria while nitrite is further reduced to nitric oxide in acidic and hypoxic tissues. L-arginine also produces NO and L-citrulline via nitric oxide synthase. VSM: Vascular Smooth Muscle

Pathways implicated in nitric oxide production

The production of NO has been classified under two distinct pathways (Figure 1). The first is an oxygen dependent pathway: L-arginine-nitric oxide (30,31). This pathway allows synthesis of nitric oxide and L-citrulline from the oxidation of L-arginine by nitric oxide synthase (NOS) enzymes. Three different isoforms that generate NO have been previously found, including: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) enzymes (10).

The second pathway for NO generation is an oxygen independent pathway: nitrate-nitrite-nitric oxide (6,32,33). The oxygen independent pathway provides an important complement to the L-arginine-nitric oxide pathway especially under circumstances of hypoxia and/or impaired NOS availability (33–37). Moreover, during exercise, the nitrate-nitrite-nitric oxide pathway has been implicated in reduced oxygen cost and increased mitochondrial efficiency over a number of sub-maximal workloads (38–42). Exploitation of this pathway may provide potential ergogenic effects for exercise activities conducted during conditions of hypoxia such as altitude or pathology (coronary artery disease or congestive heart failure).

Because oxygen is required for NO synthesis via the L-arginine-nitric oxide pathway, during incidence of ischemia or hypoxia, drugs which increase NOS activity may be less efficacious (43–45) and the nitrate-nitrite-nitric oxide pathway may become enhanced during reduced oxygen availability (33,35). As a result, increased NO production via nitrate/nitrite availability may provide a key complement during incidence of reduced oxygen tensions. Therefore, during acute hypoxia (as encountered during unacclimatized high altitude exposure), it is hypothesized that inorganic nitrate supplementation may reduce incidence of ischemia by activating NO production via the nitrate-nitrite-nitric oxide pathway. In addition, because increased plasma nitrite concomitantly reduces oxygen cost during submaximal activity, it is possible that nitrate supplementation will improve performance despite the reduced oxygen partial pressure.

Consuming dietary nitrate may result in increased NO formation as nitrate consumption has previously been shown to

increase plasma nitrite (46–48) (an *in vivo* marker of NO production (49,50)). Increased NO formation during incidence of hypoxia may negate some of the negative consequences of the reduced oxygen diffusion gradient between blood and tissues.

NO production from Nitrate and Nitrite

Both the L-arginine-NO pathway (44) and the diet (51,52) contribute to elevated plasma nitrate (NO_3^-) and nitrite (NO_2^-) levels in the body. An increase in plasma NO_3^- occurs in response to consumption of either whole foods and/or nitrate salts ($NaNO_3^-$) which contain high levels of NO_3^-. Natural foods high in NO_3^- content include the following: celery, cress, chervil, lettuce, red beetroot, spinach, and rocket (rucola) (51). Meats that have been cured or processed may contain NO_2^- as an additive to inhibit bacterial growth. In addition, NO_2^- is a product of endogenous NO oxidation and NO_3^- reduction.

Inorganic NO_3^- from dietary intake forms NO_2^- after interacting with facultative anaerobic bacteria in the mouth (53,54); this process is also referred to as bacterial nitrate reductase activity (32). Subsequently, once NO_2^- swallowed, it is converted to NO within the acidic stomach (55). This is contrary to original conclusions which postulated NO_2^- and NO_3^- were endogenously inert end products of NO (56). It is now clear that NO_3^- and NO_2^- provide an auxiliary oxygen independent pathway for NO formation. This may be especially important under conditions of hypoxia (i.e. intense or near maximal exercise) where the oxygen dependant L-arginine route may be limited by the decreased bioavailability of NOS, nicotinamide adenine dinucleotide phosphate (NADHP), flavin adenine dinucleotide (FAD) and other related co-factors (31). In addition, during incidence of hypoxia and ischemia, NO_2^- has a demonstrated capacity to reduce tissue injury (35,57–59) and inhibit the generation of reactive oxygen species (ROS)(60).

Nitrate is absorbed directly from the gastrointestinal tract with plasma NO_3^- levels reaching their peak 60 minutes after ingestion (61). After either $NaNO_3^-$ or inorganic NO_3^- ingestion, plasma NO_2^- increases (38,62–64). The initial reduction of NO_3^- to NO_2^- occurs in the mouth. Therefore, an antibacterial mouthwash administered prior to NO_3^- ingestion, attenuates the downstream conversion of NO_3^- to NO_2^-. Antibacterial mouthwash results in the removal of the commensal oral bacteria and subsequent decrement in nitrate reductase activity (65).

An increase in plasma NO_3^- and NO_2^- may increase NO production even when NOS and eNOS expression are limited by hypoxia or related oxidative stress (66,67). Dietary intake which elevates NO_2^- and NO_3^- plasma concentration, also increases NO synthesis over a wide range of exercise intensities. During high intensity exercise or instances of hypoxia, NO production via the NOS catalyst may be compromised due to reduction in the oxygen substrate (68). Further, the alternative NO_3^- to NO_2^- to NO pathway facilitates activity at altitude which is predicated with reduced partial pressure and a concomitant reliance on oxygen independent NO production (69).

High Altitude and Plasma Nitrite concentration

Individuals residing at high altitude may have greater plasma nitrite values than lowlanders. Tibetans residing at 4200m show greater bioactive NO products of plasma and red blood cell NO_3^- as well as increased plasma NO_2^- compared to residents at 206m (70). This suggests NO production was increased in highlanders alongside a greater forearm blood flow and lower vascular resistance compared to residents near sea-

level. (70). Dietary intake was monitored in the previously mentioned study and dietary nitrate was "not at a level expected to significantly increase circulating nitrate or nitrite" (70). Further, acclimatization to high altitude results in increased plasma biomarkers of NO production (NO_3^- and NO_2^-) as well as elevated cGMP indicating increased NO activity (71). In fact, enhanced circulatory extraction of NO_2^- occurs as hypoxia increases (increasing altitude) (71) which may explain the importance of the oxygen independent conversion of plasma NO_2^- to NO.

NO_2^- can also be converted to NO via allosteric NO_2^- reduction by hemoglobin as evidenced by the formation of iron-nitrosylated hemoglobin. *In vivo*, infusion of nitrite both with and without a NOS inhibitor present resulted in increased forearm blood flow during exercise (72). This points toward the complementary nitrate-nitrite-nitric oxide pathway when production of NO via NOS is impaired. Further, there was an inverse relationship between iron-nitrosylated hemoglobin formation and the oxyhemoglobin saturation ($r = –0.7$ and $P < 0.0001$) (72). The maximal rate of NO conversion from NO_2^- (via deoxyhemoglobin) occurs when hemoglobin is 50% saturated (32). In support of this finding, Shiva and colleagues (73) noted deoxymyoglobin reduces NO_2^- to NO approximately 36 times faster than deoxyhemoglobin.

High Altitude and Blood Flow

Sea level residents acclimatizing to high altitude also incur a reduction in blood flow within microcirculatory blood vessels <50μm in diameter (74). Similar reductions in the blood flow of microcirculatory small (<25 μm) and medium (26–50 μm) blood vessels was also found in lowlanders exposed to altitude (>3500m) with an explanation that the cause was perhaps due to the decreased hematocrit (reduced plasma volume) and the subsequent increase in blood viscosity (75). The authors add that the reduced blood flow may aid in oxygen delivery as the time for diffusion is increased at the capillary bed and is therefore a favorable adaptation in lowlanders acclimatizing to high altitude (75). Participants who supplement with inorganic nitrate may therefore increase plasma NO_2^- and subsequently increase the availability of NO in light of the reduced NO production from L-arginine (32).

As early as 15 minutes of acute altitude exposure (76), systemic vasodilation occurs, in part, mediated by NO production to ensure adequate oxygenation of tissues. In lowlanders acclimatized to high altitude (>3500m), both the production and availability of NO is enhanced (71). Conversely, in the pulmonary circuit, acute altitude exposure results in hypoxic pulmonary vasoconstriction. In compensation for the hypoxic encounter, alveolar ventilation increases to offset hypoxemia thereby resulting in respiratory alkalosis (77). The increased pulmonary vasoconstriction can increase pulmonary capillary pressure which increases capillary leakage leading to high altitude pulmonary edema (HAPE) (78). Interestingly, pulmonary artery systolic blood pressure was greatest in individuals who developed HAPE after altitude exposure at 4959m (measurements made over 2 day sojourn). Importantly, those individuals not exhibiting HAPE criteria had greater concentrations of nitrate-nitrite, measured via bronchoalveolar lavage fluid within one day of exposure to 4959m (79). Additionally, the concentration of expired NO was found to be lowest in individuals susceptible to HAPE during exposure to altitude (at 12, 24, 36, and 48 hours at 4,559 m); indicative of a dysfunction in pulmonary NO synthesis (80). Therefore, impaired pulmonary epithelial NO synthesis may result in decreased

bioavailability of NO, and increased pulmonary vasoconstriction may predicate the susceptibility of developing HAPE.

Given NO's effect on vascular tone and mitochondrial efficiency, greater NO availability during altitude exposure has increasing importance as ROS production increases. During altitude acclimatization, NO production/availability is associated with circulating elevations in cGMP concentrations (in the absence of changes in natriuretic peptide levels) (71). As reported by Levett (71), elevations in cGMP at 5,300m were positively correlated with microvascular blood flow in small (<25 mm diameter) and medium-sized (26–50 mm) vessels ($p=0.06$ and $p=0.025$ respectively; r value not reported), yet the cGMP concentrations were insufficient to normalize microcirculatory blood velocity at 5,300m (71). During altitude exposure, pharmacologic intervention such as with tadalafil or sildenafil (both phosphodiesterase-5 inhibitors) inhibit cGMP degradation, thereby increasing cGMP and subsequently preventing the onset of HAPE (81,82).

Critique

It is hypothesized that during exposure to high altitude, maximal exercise intensity, or cardiovascular pathology such as associated with stroke, inorganic nitrate supplementation may reduce incidence of ischemia and improve performance by activating NO production via the nitrate-nitrite-nitric oxide pathway thereby reducing oxygen cost. The NO production via the nitrate-nitrite-NO pathway acts complementary to the L-arginine pathway and may, therefore, increase NO bioavailability. In addition, nitrate supplementation has previously been shown to increase mitochondrial efficiency in humans at sea level; however, the benefits at altitude are less well known.

Increased plasma NO_2^- concentration occurs both during altitude acclimatization (>3500m) (71) and during short term (3 day) dietary nitrate supplementation (62). During exercise in conditions of reduced oxygen partial pressures, increased ROS may result. NO_3^- supplementation increases NO_2^-, and NO_2^- has been reported to have cytoprotective capabilities (i.e. improved mitochondrial oxidative phosphorylation) and to reduce mitochondrial ROS generation (60). NO_2^- has been previously established as a reservoir for NO (72) during hypoxia as NO_2^- is converted to NO (45).

While a number of physiological adaptations (increased oxygen carrying capacity, mitochondrial density, ventilatory response etc.) occur in response to altitude acclimatization, these particular changes occurring in response to NO_3^- supplementation have not been researched. Altitude acclimatization induces a number of hematological and non-hematological changes to improve performance/tolerance during hypoxia. However, the same physical changes have not been reported during short term NO_3^- supplementation (<15 days). Therefore, performance benefits similar to those resulting from altitude acclimatization may not be as likely despite the increased NO_2^- and NO bioavailability.

The limited research on dietary NO_3^- and its influence on cGMP is equivocal. NO_3^- supplementation has been shown to increase cGMP (83,84), but there is also evidence that suggests dietary NO_3^- has no influence on plasma levels of cGMP (42,62). Research supporting NO_3^- supplementation and resultant increases in cGMP at altitude is currently unavailable and is therefore, an area open for future research. Examination of soluble guanylyl cyclase activation during hypoxia could provide an explanation for the benefit of increased bioactivation of NO via NO_3^- supplementation. Mice lacking NO-sensitive guanylyl cyclase exhibited increased

hypertension at rest due to the inability to appropriately vasodilate (85). Therefore, regardless if NO production is normal, when guanalyl cyclase loses sensitivity to NO or is unable to bind/interact with NO, vascular tone impairments may result in reduced blood flow/oxygen flux to working muscles. Such a condition may be a primary component of reduced performance during exercise at altitude.

It is possible that an overabundance of NO via nitrate supplementation (elevated plasma nitrite) could induce receptor desensitization or hypotension, a negative consequence. While NO_3^- supplementation has been shown to reduce blood pressure (83), these effects appear to be therapeutic in both healthy patients (i.e. reducing blood pressure but not reported to induce problematic hypotension)(63,86) and patients with peripheral artery disease (64). In fact, the consumption of natural sources of dietary nitrate, including certain fruits and vegetables (51), has been generally associated with decreased blood pressure, reduced oxygen cost during submaximal exercise, and increased exercise tolerance (87).

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