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The nitrate-nitrite-nitric oxide pathway: Its role in human exercise physiology

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Abstract

Nitric oxide (NO) is a potent signalling molecule that influences an array of physiological responses. It was traditionally assumed that NO was derived exclusively via the nitric oxide synthase (NOS) family of enzymes. This complex reaction requires a five electron oxidation of L-arginine and is contingent on the presence of numerous essential substrates (including O_2) and co-factors. Recently an additional, O_2-independent, NO generating pathway has been identified, where nitrite (NO_2^-) can undergo a simple one electron reduction to yield NO. NO_2^- is produced endogenously from the oxidation of NO and also from the reduction of dietary nitrate (NO_3^-) by facultative bacteria residing on the tongue. Recent data show that dietary NO_3^- supplementation, which increases the circulating plasma [NO_2^-], reduces the O_2 cost of submaximal exercise in healthy humans. This finding is striking given that efficiency during moderate-intensity exercise has been considered to be immutable. There is evidence that the muscle ATP turnover at a fixed work rate is reduced and the mitochondrial P/O ratio is increased following NO_3^- supplementation, which offers important insights into the physiological bases for the reduced $\dot{V}O_2$ during exercise. NO_3^- supplementation has also been shown to improve exercise performance in both healthy and patient populations. Therefore, dietary NO_3^- supplementation may represent a practical and cost-effective method to improve exercise efficiency and exercise tolerance in humans. Given that a NO_3^--rich diet may have numerous cardiovascular and other health benefits, dietary NO_3^- intake may have important implications for human lifelong health and performance.

Keywords: *Beetroot juice, bioenergetics, efficiency, exercise performance, fatigue, muscle metabolism*

Introduction

Nitric oxide (NO) is a gaseous physiological signalling molecule that was first recognised as a 'nitrovasodilator' capable of relaxing the vascular endothelium (Furchgott & Zawadzki, 1980; Ignarro, Buga, Wood, Byrns, & Chaudhuri, 1987; Murad, Mittal, Arnold, Katsuki, & Kimura, 1978). Today, NO is one of the most researched molecules in physiology and medicine and is known to influence a wide array of physiological processes including: skeletal muscle glucose uptake (Merry, Lynch, & McConell, 2010), neurotransmission (Garthwaite, 2008), sarcoplasmic reticulum (SR) calcium (Ca^{2+}) handling (Hart & Dulhunty, 2000; Viner, Williams, & Schoneich, 2002), mitochondrial respiration (Brown & Cooper, 1994) and skeletal muscle fatigue (Percival, Anderson, Huang, Adams, & Froehner, 2010). The production of NO by the NO synthase (NOS) enzymes is well documented with endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms of this enzyme having been identified (Stamler & Meissner, 2001). These enzymes catalyse the complex five electron oxidation of L-arginine to yield NO and L-citrulline in a reaction requiring oxygen (O_2), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), haem and calmodulin as substrates/co-factors (Alderton, Cooper, & Knowles, 2001; Figure 1). A reduced bioavailability of any of these essential components compromises the production of NO through the NOS pathway (e.g. Crabtree, Tatham, Hale, Alp, & Channon, 2009). NOS-derived NO is known to be lower in cardiovascular

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(Förstermann, 2010) and metabolic (Wu & Meininger, 2009) disease, and recent evidence indicates that humans with impaired NO synthesis have poor exercise tolerance (Lauer et al., 2008). Thus, effective NO synthesis is obligatory for normal physiological functioning and exercise tolerance.

More recently, an additional NO generating pathway has been identified (Benjamin et al., 1994; Lundberg, Weitzberg, Lundberg, & Alving, 1994) whereby NO is formed by the simple one electron reduction of nitrite (NO_2^-; Figure 1). This NO generating pathway is important as it represents a means to increase NO production when NO synthesis by the NOS enzymes is impaired (Bryan, Calvert, Gundewar, & Lefer, 2008; Calström et al., 2010). The potentially important role of this additional NO pathway in ischaemia-reperfusion injury (Raat, Shiva, & Gladwin, 2009), hypertension (Gilchrist, Shore, & Benjamin, 2011) and cardiovascular health (Lundberg, Calström, Larsen, & Weitzberg, 2011) has been the subject of a number of excellent reviews published elsewhere and will not be addressed in detail here. Instead, this review will summarise the recent findings that human muscle metabolism, exercise efficiency and exercise performance may be positively impacted by dietary nitrate (NO_3^-)

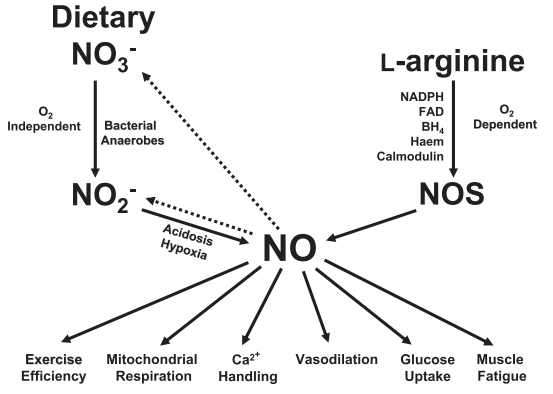

Figure 1. The pathways of nitric oxide (NO) generation in humans. The right branch of the schematic represents the conventional L-arginine-NOS-NO pathway while the left branch of the schematic represents the NO_3^--NO_2^--NO pathway. The NOS-NO pathway requires L-arginine, O_2 and NADPH as essential substrates as well as the co-factors: FAD, FMN, BH_4, haem and calmodulin. In the O_2^- independent NO_3^--NO_2^--NO pathway, commensal anaerobes reduce NO_3^- to NO_2^- in the mouth with the ingested NO_2^- further reduced to NO in acidic and hypoxic tissues. Some of the physiological processes regulated by NO include: exercise efficiency, mitochondrial respiration, sarcoplasmic reticulum Ca^{2+} handling, vasodilation, skeletal muscle glucose uptake and muscle fatigue. However, the free radical behaviour of NO limits its half-life in vivo. Importantly, a portion of the NO oxidation products, NO_3^-, produced through the reaction between NO and oxyhaemoglobin, and NO_2^-, produced by the oxidation of NO by ceruloplasmin, can be recycled back to NO via the NO_3^--NO_2^--NO pathway, as indicated by the dashed arrows. This dual pathway for NO synthesis facilitates NO production across a range of physiological conditions, including exercise.

supplementation (Table I). The candidate physiological mechanisms as well as the therapeutic and practical implications of these findings will be discussed. Finally, key areas for future research will be identified to stimulate further investigation in this field.

The NO_3^--NO_2^--NO pathway

In humans NO_3^- is formed through the reaction of NO_2^- (Kosaka & Tyuma, 1987) or NO with oxyhaemoglobin (Cooper, 1999), whilst NO_2^- can be formed by the reaction of NO with molecular O_2 (Ignarro, Fukuto, Griscavage, Rogers, & Byrns, 1993) or the oxidised copper active site of cytochrome *c* oxidase (Cooper, Torres, Sharpe, & Wilson, 1997), and also from the oxidation of NO by ceruloplasmin (Shiva et al., 2006). It was traditionally assumed that NO_3^- and NO_2^- were inert derivatives of NO production (Moncada & Higgs, 1993). However, a substantial body of evidence has now demonstrated that this is not the case and that, in fact, these NO metabolites can be recycled back into bioactive NO under certain physiological conditions (Bryan, 2006; Lundberg & Weitzberg, 2009, 2010; Lundberg, Weitzberg, Cole, & Benjamin, 2004; van Faassen et al., 2009; Figure 1).

NO_3^- is ingested as part of a healthy diet (Bryan & Hord, 2010a). Vegetables account for 60–80% of the daily NO_3^- intake in a western diet (Ysart et al., 1999) and green leafy vegetables such as lettuce, spinach and beetroot are particularly rich in NO_3^- (Bryan & Hord, 2010a). Ingested inorganic NO_3^- is rapidly absorbed from the gut and passes into the systemic circulation with peak plasma [NO_3^-] observed ~60 minutes following NO_3^- ingestion (Lundberg & Weitzberg, 2009, 2010). While ~60% of systemic NO_3^- is excreted in the urine (Lundberg & Weitzberg, 2009, 2010), ~25% of NO_3^- passes into the enterosalivary circulation and is concentrated in saliva at least 10-fold (Lundberg & Govoni, 2004). In the mouth, facultative anaerobic bacteria on the surface of the tongue reduce NO_3^- to NO_2^- (Duncan et al., 1995). This NO_2^- is swallowed and reduced to NO and other reactive nitrogen intermediates within the acidic environment of the stomach (Benjamin et al., 1994; Lundberg et al., 2004). However, it is clear that some NO_2^- is absorbed to increase circulating plasma [NO_2^-] (Dejam, Hunter, Schechter, & Gladwin, 2004; Lundberg & Govoni, 2004). Therefore, dietary NO_3^- supplementation represents a practical method to increase the circulating plasma [NO_2^-]. This has been demonstrated in humans after pharmacological sodium nitrate ($NaNO_3^-$) (Larsen, Weitzberg, Lundberg, & Ekblom, 2007, 2010; Larsen et al., 2011; Lundberg & Govoni, 2004) and potassium nitrate (KNO_3^-) (Kapil et al., 2010) ingestion, as well

Table I. The effect of nitrate supplementation on exercise efficiency and exercise performance

Author	Participants	NO_3^- administration and exercise protocol	NO indices	Physiological and performance changes
Larsen et al. (2007)	Trained males ($\dot{V}O_{2max}$ 55 ± 3 ml kg^{-1} min^{-1})	3 days NaNO3 − supplementation (0.1 mmol kg^{-1} day^{-1}) 5 minutes cycling at work rates equivalent to 45, 60, 70, 80, 85 and 100% $\dot{V}O_{2max}$	↑ in plasma [NO_3^-] ↑ in plasma [NO_2^-] ↓ in SBP	↓ $\dot{V}O_2$ over 4 lowest WRs ↑ average GE over 4 lowest WRs ↑ average DE over 4 lowest WRs
Bailey et al. (2009b)	Recreationally active males ($\dot{V}O_{2max}$ 49 ± 5 ml kg^{-1} min^{-1})	6 days of NO_3^--rich beetroot juice supplementation (~5.6 mmol NO_3^-·day^{-1}). A total of 4 MI and 2 SI cycle exercise bouts were completed on days 4–6 of supplementation	↑ in plasma [NO_2^-] ↓ in SBP	↓ $\dot{V}O_2$ amplitude during MI ↓ $\dot{V}O_2$ slow component during SI ↓[HHb] ↑[HbO_2] ↑[Hb_{tot}] ↑ severe exercise Tlim
Larsen et al. (2010)	Healthy recreationally active males and females	Acute (1 hour prior to exercise) ingestion of 0.1 mmol kg^{-1} of $NaNO_3^-$) prior to LI cycle exercise 2 days $NaNO_3^-$ supplementation (0.1 mmol kg^{-1}·day^{-1}) prior to a combined arm and leg cycle IT	↑ in plasma [NO_3^-] ↑ in plasma [NO_2^-] ↑ in plasma cGMP	↓ $\dot{V}O_2$ during LI cycle exercise following acute ingestion ↓ $\dot{V}O_{2max}$ following 2 days supplementation Tendency for improved Tlim during IT
Bailey et al. (2010)	Recreationally active males	6 days of NO_3^--rich beetroot juice supplementation (~5.1 mmol NO_3^-·day^{-1}). A total of 6 LI and 3 HI two-legged knee-extensor exercise bouts were completed on days 4–6 of supplementation	↑ in plasma [NO_2^-] ↓ in SBP ↓ in DBP ↓ in MAP	↓ $\dot{V}O_2$ amplitude during LI ↓ $\dot{V}O_2$ slow component during HI ↓ muscle ATP turnover rate ↓ muscle ADP accumulation ↓ muscle P_i accumulation ↓ muscle PCr depletion ↑ severe exercise Tlim
Vanhatalo et al. (2010)	Recreationally active males and females ($\dot{V}O_{2max}$ 47 ± 8 ml kg^{-1} min^{-1})	Acute (2.5 hours prior to exercise) and 5 and 15 days of supplementation with NO_3^--rich beetroot juice supplementation (~5.2 mmol NO_3^-·day^{-1}). 2 bouts of MI and 1 IT until Tlim after 2.5 hours and 5 and 15 days of NO_3^- supplementation	After 2.5 hours and 5 and 15 days of NO_3^- supplementation: ↑ in plasma [NO_2^-] ↓ in SBP ↓ in MAP	After 2.5 hours and 5 and 15 days: ↓ in $\dot{V}O_2$ amplitude during MI After 15 days: ↑ $\dot{V}O_{2max}$ ↑ PWR in IT ↑ GET WR
Larsen et al. (2011)	Recreationally active males and females ($\dot{V}O_{2max}$ 56 ± 3 ml kg^{-1} min^{-1})	3 days $NaNO_3^-$ supplementation (0.1 mmol kg^{-1}·day^{-1}) LI cycle exercise	↑ in plasma [NO_3^-] ↑ in plasma [NO_2^-]	↓ $\dot{V}O_2$ during LI cycle exercise ↑ mitochondrial P/O ratio
Lansley et al. (2011b)	Recreationally active males ($\dot{V}O_{2max}$ 55 ± 7 ml kg^{-1} min^{-1})	6 days of NO_3^--rich beetroot juice supplementation (~6.2 mmol NO_3^-·day^{-1}). A total of 4 moderate and 2 severe treadmill running bouts were completed on days 4–6 of supplementation	↑ in plasma [NO_2^-] ↓ in SBP	↓ $\dot{V}O_2$ during walking ↓ $\dot{V}O_2$ amplitude during MI ↓ EE $\dot{V}O_2$ during SI ↑ in severe exercise Tlim
Lansley et al. (2011a)	Competitive male cyclists ($\dot{V}O_{2max}$ 56 ± 6 ml kg^{-1} min^{-1})	Acute (2–2.5 hours prior to exercise) ingestion of NO_3^-rich beetroot juice supplementation (~6.2 mmol NO_3^-). 4- and 16.1-km cycling TT	↑ in plasma [NO_2^-] ↓ in SBP	Improved 4- and 16.1-km TT performance

Table I (*Continued*)

Author	Participants	NO_3^- administration and exercise protocol	NO indices	Physiological and performance changes
Kenjale et al. (2011)	PAD patients	Acute (3 hour prior to exercise) ingestion of NO_3^--rich beetroot juice (~9 mmol NO_3^-) IT until Tlim	↑ in plasma $[NO_3^-]$ ↑ in plasma $[NO_2^-]$ ↓ in DBP	↓ $\dot{V}O_2$ during walking ↓[HHb] ↑$[HbO_2]$ ↑$[Hb_{tot}]$ ↑ Tlim
Bescós et al. (2011)	Trained male cyclists and triathletes ($\dot{V}O_{2max}$ 55 ± 3 ml kg^{-1} min^{-1})	Acute (3 hour prior to exercise) ingestion of 10 mg kg^{-1} of $NaNO_3^-$) 6 minutes cycling at work rates equivalent to 2, 2.5, 3 and 3.5 W·kg^{-1} body mass, followed by an IT until Tlim	↑ in plasma $[NO_3^-]$ ↑ in plasma $[NO_2^-]$	↔ $\dot{V}O_2$ over submaximal WRs ↓ $\dot{V}O_{2max}$ ↔ IT Tlim

↑, significant increase; ↓, significant reduction; ↔, no significant difference; $\dot{V}O_{2max}$, maximum oxygen uptake; $NaNO_3^-$, sodium nitrate; SBP, systolic blood pressure; $[NO_3^-]$, nitrate concentration; $[NO_2^-]$, nitrite concentration; $\dot{V}O_2$, oxygen uptake; GE, gross efficiency; DE, delta efficiency; WRs, work rates; MI, moderate-intensity; LI, low-intensity; SI, severe-intensity; [HHb], deoxyhaemoglobin concentration; $[O_2Hb]$, oxyhaemoglobin concentration; $[Hb_{tot}]$, total haemoglobin concentration; Tlim, time until the limit of tolerance; DBP, diastolic blood pressure; MAP, mean arterial pressure; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; PCr, phosphocreatine; IT, incremental test; PWR, peak work rate; GET, gas exchange threshold; WR, work rate; cGMP, cyclic guanosine monophosphate; P/O ratio, ADP/oxygen ratio; TT, time trial; EE, end exercise; PAD, peripheral arterial disease.

as following NO_3^--rich beetroot juice ingestion (Bailey et al., 2009b, 2010; Lansley et al., 2011a, 2011b; Vanhatalo et al., 2010; Webb et al., 2008). It is important to note, however, that the characteristic rise in plasma $[NO_2^-]$ following an oral NO_3^- bolus is largely abolished after antibacterial mouthwash treatment (Govoni, Jansson, Weitzberg, & Lundberg, 2008), indicating that the reduction of NO_3^- to NO_2^- in humans is critically dependent on the bacterial NO_3^- reductases.

The final step in the NO_3^--NO_2^--NO pathway is the one electron reduction of NO_2^- to NO. This NO_2^- reduction is catalysed by deoxyhaemoglobin (Cosby et al., 2003), deoxymyoglobin (Shiva et al., 2007a), NOS (Vanin, Bevers, Slama-Schwok, & van Faassen, 2007), xanthine oxidase (Zhang et al., 1998), aldehyde oxidase (Li, Cui, Kundu, Alzawahra, & Zweier, 2008), cytochrome P-450 (Kozlov, Dietrich, & Nohl, 2003) and the mitochondrial electron transfer complexes (Kozlov, Staniek, & Nohl, 1999). This reaction is potentiated in hypoxic (Castello, David, McClure, Crook, & Poyton, 2006) and acidic (Modin et al., 2001) environments such as those which may be extant during exercise (Bailey et al., 2010; Richardson, Leigh, Wagner, & Noyszewski, 1999). The existence of an alternative NO generation pathway is important as it promotes NO synthesis under conditions that would otherwise limit the production of NO from NOS, including hypoxia (Stuehr, Santolini, Wang, Wei, & Adak, 2004) and oxidative stress (Huang, Xiao, Samii, Vita, & Keaney, 2001; Jaimes, Sweeney, & Raij, 2001). Importantly, contracting skeletal muscles become hypoxic (Richardson et al., 1999) and produce reactive oxygen species at an elevated rate (McArdle, Pattwell, Vasilaki, McArdle, & Jackson, 2005). This suggests that the NO_3^--NO_2^--NO pathway may be particularly important for NO production during exercise. This compensatory role of the NO_3^--NO_2^--NO pathway is supported by the observations that dietary supplementation with NO_2^- (Bryan et al., 2008) and NO_3^- (Calström et al., 2010) restores tissue and plasma $[NO_3^-]$ and $[NO_2^-]$ (markers of NO synthesis) in eNOS knockout mice. Therefore, the complementary nature of the NOS-NO and NO_3^--NO_2^--NO pathways ensures that NO synthesis can occur across a wide range of cellular O_2 tensions and redox states. It is important to note, however, that NO_2^- may modify haem groups by nitrosylation and protein thiols by S-nitrosation (Bryan et al., 2005) suggesting that NO_2^- may induce physiological effects independent of its reduction to NO.

Exercise efficiency

In 2007, Larsen, Weitzberg, Lundberg and Ekblom reported that three days of $NaNO_3^-$ supplementation

increased plasma [NO_2^-] and reduced the O_2 cost of submaximal cycle exercise in humans (Larsen et al., 2007; Table I). Blood [lactate], heart rate and minute ventilation ($\dot{V}_E$) were not significantly impacted by $NaNO_3^-$ supplementation; however, the underlying mechanisms for the action of $NaNO_3^-$ and how $NaNO_3^-$ administration influenced the dynamics of oxidative metabolism were not investigated in this study. Since the authors did not investigate the effects of $NaNO_3^-$ supplementation on exercise performance, it was also unclear whether NO_3^- ingestion could be ergogenic. The initial findings of Larsen et al. (2007) were corroborated in the study of Bailey et al. (2009b) in which NO_3^- was administered in the form of beetroot juice. Bailey et al. administered NO_3^- as NO_3^--rich beetroot juice because human ingestion of pharmacological $NaNO_3^-$ is restricted in the UK. Following three days of beetroot juice supplementation the plasma [NO_2^-] was doubled, and the steady-state $\dot{V}O_2$ during moderate-intensity constant-work-rate cycle exercise (performed below the gas exchange threshold) and the $\dot{V}O_2$ 'slow component' during severe-intensity constant-work-rate exercise (performed above the critical power) were reduced (Bailey et al., 2009b; Table I; Figure 2). In addition, the authors used near-infrared spectroscopy to assess muscle oxygenation and reported that the [oxyhaemoglobin] and total [haemoglobin] in the area of interrogation within the *m. vastus latealis* were increased after NO_3^- supplementation. The [deoxyhaemoglobin] amplitude, a non-invasive index of muscle fractional O_2 extraction (Grassi et al., 2003), was reduced following NO_3^- supplementation (Bailey et al., 2009b). The O_2 cost of moderate-intensity

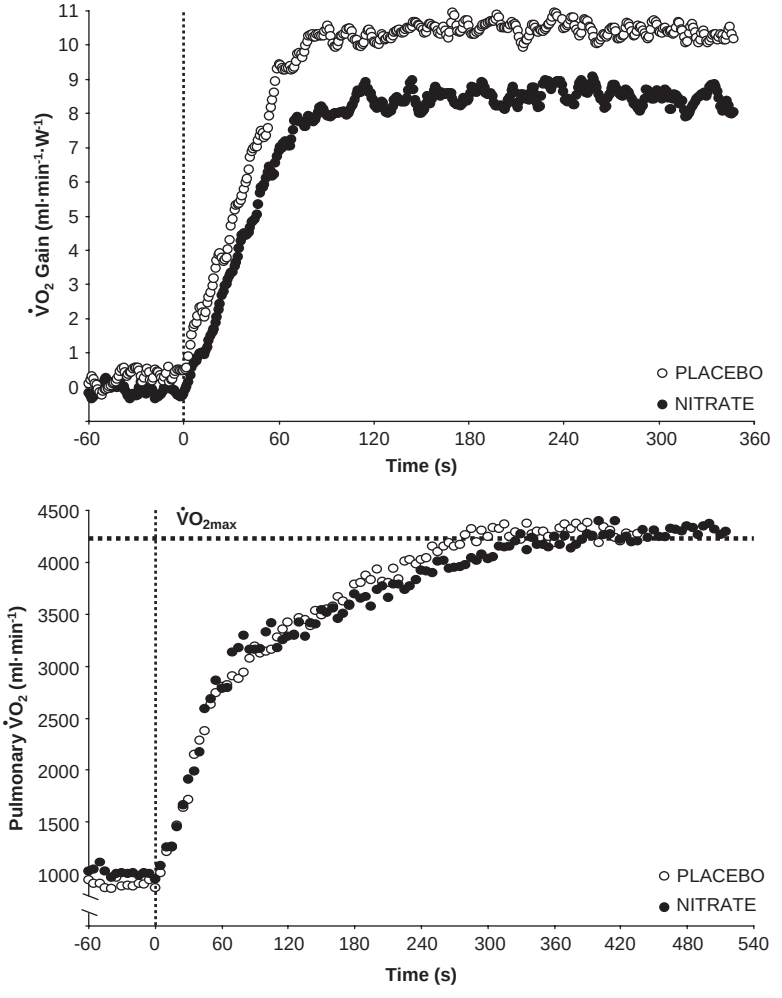

Figure 2. Group mean pulmonary oxygen uptake ($\dot{V}O_2$) 'gain' during moderate-intensity cycle ergometry (*upper panel*) and a pulmonary $\dot{V}O_2$ trace for a representative individual during severe-intensity cycle exercise continued until the limit of tolerance (*lower panel*) following nitrate and placebo supplementation. The $\dot{V}O_2$ gain is calculated by dividing the change in $\dot{V}O_2$ (from baseline) by the change in work rate during a step exercise test. Data are presented as the average of four repeated moderate cycle exercise bouts. The dashed vertical line represents the abrupt imposition of the work rate from a baseline of 'unloaded' cycling. The dashed horizontal line represents the $\dot{V}O_{2\,max}$ of the representative individual. Note the significant reduction in the $\dot{V}O_2$ gain, which is the reciprocal of delta efficiency, with nitrate supplementation. Note also the reduced $\dot{V}O_2$ 'slow component', delayed attainment of the $\dot{V}O_{2\,max}$ and extended tolerable duration of severe-intensity cycling exercise following nitrate supplementation. Data are redrawn from Bailey et al. (2009b).

cycle exercise is considered to be essentially independent of factors such as age, health status and physical fitness (Jones & Poole, 2005) and is unaffected by exercise training (Bailey, Wilkerson, DiMenna, & Jones, 2009a), prior exercise (Burnley, Jones, Carter, & Doust, 2000), increasing muscle O_2 delivery by breathing an O_2 enriched gas mixture (Wilkerson, Berger, & Jones, 2006) or erythropoietin administration (Wilkerson, Rittweger, Berger, Naish, & Jones, 2005), and intravenous antioxidant infusion (Bailey et al., 2011). As such, it is striking that a short-term, natural dietary intervention can improve the efficiency of muscular work (Figure 2).

The reduction in steady-state $\dot{V}O_2$ after NO_3^- supplementation was in the order of 3–5% in the studies of Larsen et al. (2007) and Bailey et al. (2009b). A similar reduction in steady-state $\dot{V}O_2$ during moderate-intensity cycle ergometry has been reported following acute NO_3^- treatment: 60 minutes following $NaNO_3^-$ administration (Larsen et al., 2010) and 2.5 hours following beetroot juice ingestion (Vanhatalo et al., 2010; Table I). The improved moderate exercise efficiency was sustained when NO_3^- supplementation was continued for 15 days (Vanhatalo et al., 2010). This indicates that longer term NO_3^- supplementation does not elicit greater improvements in exercise efficiency but, importantly, that tolerance to the intervention does not develop (at least up to 15 days). The reduction in $\dot{V}O_2$ following NO_3^- administration is not unique to cycling exercise, having also been observed during two-legged knee-extensor exercise (Bailey et al., 2010) and treadmill walking and running (Lansley et al., 2011b; Table I). Importantly, Lansley et al. (2011b) did not observe a reduction in $\dot{V}O_2$ when the subjects were supplemented with beetroot juice that had been depleted of NO_3^- using an ion-exchange resin. This confirms that NO_3^- is the key 'active' ingredient responsible for the physiological changes observed following beetroot juice supplementation. It does not rule out, however, a synergistic role for other components of beetroot juice such as antioxidants and polyphenols which may facilitate the reduction of NO_3^- to NO_2^- and NO (Carlsson, Wiklund, Engstrand, Weitzberg, & Lundberg, 2001; Gago, Lundberg, Barbosa, & Laranjinha, 2007). Collectively, these results indicate that the reduced $\dot{V}O_2$ following NO_3^- supplementation is reproducible and can be observed across a range of different supplementation regimes and exercise modalities.

An improved exercise efficiency has been consistently reported when recreationally active humans ($\dot{V}O_{2\,max}$ values typically between 45 and 55 ml $kg^{-1} \cdot min^{-1}$) have been supplemented with NO_3^- (Bailey et al., 2009b, 2010; Lansley et al., 2011b; Larsen et al., 2007, 2010, 2011; Vanhatalo et al., 2010; Table I). However, Bescós et al. (2011) recently reported that acute $NaNO_3^-$ ingestion did not significantly improve submaximal exercise efficiency in trained subjects ($\dot{V}O_{2\,max}$ of 65 ml kg^{-1} min^{-1}). $NaNO_3^-$ administration reduced $\dot{V}O_2$ during the four submaximal cycling work rates by $\sim$2–3% but this difference did not attain statistical significance (Bescós et al., 2011; Table I). Perhaps importantly, plasma $[NO_2^-]$ was only increased by 16% in this study, whereas previous studies have observed far greater increases in plasma $[NO_2^-]$ following NO_3^- supplementation, in the order of 100% (Bailey et al., 2009b, 2010; Lansley et al., 2011b; Larsen et al., 2007, 2010, 2011; Vanhatalo et al., 2010). The resting plasma $[NO_3^-]$ (Jungersten, Ambring, Wall, & Wennmalm, 1997) and plasma $[NO_3^-] + [NO_2^-]$ ([NOx]) (Schena, Cuzzolin, Rossi, Pasetto, & Benoni, 2002) are higher in athletes compared to non-athletic controls, which may reduce the scope for NO_3^- supplementation to improve exercise efficiency in this population. Alternatively, athletes may require a larger NO_3^- dose to elicit similar changes in plasma $[NO_2^-]$ and exercise efficiency to those observed in recreationally active participants. Further research is needed to elucidate the influence of NO_3^- supplementation on exercise efficiency in athletes.

Exercise performance

Plasma $[NO_2^-]$ has recently been identified as an important correlate of exercise tolerance in healthy humans (Dreissigacker, Wendt, Wittke, Tsikas, & Maassen, 2010; Rassaf et al., 2007). Given that NO_3^- supplementation increases plasma $[NO_2^-]$, this intervention may therefore have the potential to improve exercise tolerance. This hypothesis was tested in the study of Bailey et al. (2009b). Plasma $[NO_2^-]$ was doubled and exercise tolerance was enhanced by 16% following NO_3^--rich beetroot juice supplementation (Bailey et al., 2009b; Figure 2) suggesting that NO_3^- supplementation may indeed be ergogenic. Subsequent experiments have reported improvements in exercise tolerance of 25% during two-legged knee-extensor exercise (Bailey et al., 2010), and of 15% during treadmill running (Lansley et al., 2011b) following 6 days of beetroot juice supplementation. Improved incremental exercise performance has also been noted following 6 days of beetroot juice supplementation during single-legged knee extension exercise (Lansley et al., 2011b) and after 15 days of beetroot juice supplementation during cycle exercise (Vanhatalo et al., 2010). A trend for an improved exercise tolerance (+7%) during combined incremental arm and leg exercise has also been reported following 2 days of $NaNO_3^-$ supplementation (Larsen et al., 2010). This observation was made in concert with a reduced $\dot{V}O_{2\,max}$ (−3%) which indicated that the

subjects were more energy efficient even at maximal exertion following NO_3^- supplementation (Larsen et al., 2010). Incremental exercise performance was not significantly different (+2%) in trained athletes following acute $NaNO_3^-$ administration, despite a 4% statistically significant reduction in $\dot{V}O_{2max}$ (Bescós et al., 2011). A reduction in $\dot{V}O_{2max}$ is not always observed following NO_3^- supplementation (Bailey et al., 2009b; Vanhatalo et al., 2010; Figure 2). It is possible that the influence of NO_3^- supplementation on the $\dot{V}O_{2max}$ may be dependent on the exercise modality and/or the training status of the subjects. Further research is needed to investigate the influence of dietary NO_3^- supplementation on the $\dot{V}O_{2max}$.

Based on existing data, NO_3^- supplementation appears to predispose to improved metabolic efficiency and exercise tolerance in healthy humans (Bailey et al., 2009b, 2010; Lansley et al., 2011b; Vanhatalo et al., 2010; Table I; Figure 2). During constant-work-rate exercise, the improved exercise tolerance following NO_3^- supplementation was in the range of 16–25% (Bailey et al., 2009b, 2010; Lansley et al., 2011b). However, the magnitude of improvement in 'actual' exercise performance would be expected to be far smaller; indeed, using the predictions of Hopkins, Hawley and Burke (1999), a ~20% improvement in time to exhaustion would be expected to correspond to an improvement in exercise performance (time taken to cover a set distance) of 1–2%. This hypothesis was tested in the study of Lansley et al. (2011a) where competitive but sub-elite cyclists completed, on separate days, 4 and 16.1 km time trials following acute beetroot juice ingestion. Consistent with the experimental hypothesis, NO_3^- administration improved 4 and 16.1 km time trial performance by ~2% (Lansley et al., 2011a). These improvements in exercise performance occurred in concert with an elevated plasma [NO_2^-] (+ 136%), a higher mean power output (+5%) and an increase in the power output/$\dot{V}O_2$ ratio (Lansley et al., 2011a). Therefore, trained subjects were able to produce a higher power output for the same oxidative energy turnover (the inverse of a lower $\dot{V}O_2$ for the same power output), resulting in an improved exercise performance following NO_3^- supplementation.

Clinical populations

A characteristic feature of patients with cardiovascular diseases is exercise intolerance (e.g. Esposito, Mathieu-Costello, Shabetai, Wagner, & Richardson, 2010). This exercise intolerance occurs in concert with a reduced NO production during exercise (Adachi et al., 1997; Lauer et al., 2008). Three months of exercise training has been shown to increase plasma [NO_2^-] in peripheral arterial disease patients and this increase in plasma [NO_2^-] was positively correlated to the improvement in exercise tolerance (Allen et al., 2010). Therefore, increasing the plasma [NO_2^-] via dietary supplementation with NO_3^- may represent a practical and cost-effective intervention to restore NO homeostasis and improve exercise tolerance in these populations. This hypothesis was recently confirmed by Kenjale et al. (2011) who showed that acute NO_3^--rich beetroot juice administration increased the tolerable duration of exercise and the time before the onset of claudication in peripheral arterial disease patients performing an incremental walking test (Table I). The increase in plasma [NO_2^-] after NO_3^- administration was also positively correlated to the increase in exercise tolerance. Moreover, in line with the findings of Bailey et al. (2009b), Kenjale et al. (2011) demonstrated that beetroot juice supplementation significantly reduced pulmonary $\dot{V}O_2$ and muscle [deoxyhaemoglobin], and increased the [oxyhaemoglobin] and total [haemoglobin] signals during treadmill walking in peripheral arterial disease patients (Kenjale et al., 2011). Therefore, NO_3^- administration may have implications for improving muscle O_2 availability and energy efficiency in pathologies that compromise convective O_2 delivery. The reduced energy cost of muscular work following NO_3^- supplementation may have the potential to improve functional capacity and quality of life in clinical populations.

Mechanistic bases

One potential explanation for the reduced O_2 cost of submaximal exercise following NO_3^- supplementation could be a compensatory increase in anaerobic energy yield. Although NO_3^- administration has been shown to have no influence on blood [lactate] (Bailey et al., 2009b; Larsen et al., 2007), this crude marker of anaerobic metabolism does not convincingly exclude a role for an increased anaerobic energy contribution. Another candidate mechanism is a reduced ATP turnover to generate a given submaximal muscle power output. Alternatively, NO_3^- administration may improve exercise efficiency by reducing the O_2 cost of mitochondrial ATP resynthesis (i.e. by increasing the mitochondrial P/O ratio).

Bailey et al. (2010) investigated the first two of these mechanisms using calibrated ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS). This procedure permitted the in vivo assessment of absolute muscle concentration changes in phosphocreatine ([PCr]), inorganic phosphate ([P_i]) and adenosine diphosphate ([ADP]), as well as pH (Kemp, Meyerspeer, & Moser, 2007). The ATP supply contributed

by PCr hydrolysis, anaerobic glycolysis and oxidative phosphorylation during knee-extensor exercise was also calculated (Lanza, Wigmore, Befroy, & Kent-Braun, 2006; Layec et al., 2009). The estimated ATP turnover rates from PCr hydrolysis and oxidative phosphorylation were lower following beetroot juice supplementation, and contributed to a significant reduction in the estimated total ATP turnover rate during both low- and high-intensity exercise (Bailey et al., 2010). It is known that the ATP turnover rate in contracting myocytes is determined, in the large part, by the activity of actomyosin ATPase and Ca^{2+}-ATPase (Barclay, Woledge, & Curtin, 2007). NO has been shown to slow myosin cycling kinetics and to increase force production per power stroke (Evangelista et al., 2010) and to reduce Ca^{2+}-ATPase activity (Ishii et al., 1998). As such, elevated NO production following beetroot juice supplementation may have reduced skeletal muscle ATP turnover by reducing the activity of actomyosin ATPase and/or Ca^{2+}-ATPase. The intramuscular accumulation of ADP and P_i and the extent of PCr depletion were also blunted following NO_3^- supplementation (Bailey et al., 2010). These data indicate that dietary NO_3^- supplementation improves the coupling between ATP hydrolysis and muscle force production, implicating this as an important determinant of the reduced $\dot{V}O_2$ during exercise. Consistent with this notion, the changes in [ADP], [P_i] and [PCr] following NO_3^- supplementation would be predicted to reduce the stimuli for increasing oxidative phosphorylation based on existing models of respiratory control (Bose, French, Evans, Joubert, & Balaban, 2003; Brown, 1992; Chance & Williams, 1955; Mahler, 1985). Moreover, given that the estimated ATP turnover rate from PCr hydrolysis was significantly reduced, and the ATP turnover rate from anaerobic glycolysis was not different following NO_3^- supplementation, these data exclude the possibility than the reduced O_2 cost of muscle contraction is consequent to or compensated by increased anaerobic energy liberation.

The accumulation of metabolites such as [ADP] and [P_i] and the rate of depletion of the finite intramuscular [PCr] reserves are important contributors to muscle fatigue development (Allen, Lamb, & Westerblad, 2008; Jones, Wilkerson, DiMenna, Fulford, & Poole, 2008). While the intramuscular [ADP], [P_i] and [PCr] were similar at exhaustion in the NO_3^- and placebo conditions in the study of Bailey et al. (2010), the time taken to achieve these critical concentrations was delayed following NO_3^- supplementation and this, in part, may explain the improved exercise tolerance. In line with these data, dietary NO_3^- supplementation has been shown to reduce the development of the $\dot{V}O_2$ 'slow component' during high-intensity exercise such that the attainment of the $\dot{V}O_{2\,max}$ is delayed and the tolerable duration of exercise is extended (Bailey et al., 2009b; Figure 2). In a subsequent study, we estimated the muscle maximum oxidative capacity (Q_{max}) using ^{31}P-MRS (Lansley et al., 2011b). Short-term dietary NO_3^- supplementation did not significantly alter Q_{max} (Lansley et al., 2011b). This finding was recently corroborated by Larsen et al. (2011) who did not observe changes in markers of mitochondrial density or biogenesis after NO_3^- supplementation. Collectively, these results suggest that mitochondrial biogenesis does not contribute to the improvement in exercise tolerance with NO_3^- supplementation of up to six days. Following 15 days of NO_3^- supplementation, however, small but significant increases in both $\dot{V}O_{2\,max}$ and power output during incremental cycling exercise were observed (Vanhatalo et al., 2010). It is possible that longer term NO_3^- supplementation may promote mitochondrial biogenesis and/or angiogenesis that may elicit an increase in $\dot{V}O_{2\,max}$ and greater gains in exercise performance compared with short-term NO_3^- supplementation. Further research is needed to clarify the underlying mechanisms for the improved muscle performance following prolonged NO_3^- supplementation.

Although the data of Bailey et al. (2010) showed a reduced ATP cost of muscle force production following NO_3^- supplementation, it was not possible to exclude a role for an increased mitochondrial P/O ratio in reducing $\dot{V}O_2$ during low-intensity exercise. To determine whether NO_3^- supplementation altered the mitochondrial P/O ratio, Larsen et al. (2011) isolated mitochondria from the vastus lateralis muscle of healthy humans supplemented with $NaNO_3^-$. The resultant mitochondrial suspension was added to a reaction medium containing the substrates, pyruvate and maltate, allowing mitochondrial respiration to be investigated. With a submaximal rate of ADP infusion, which was selected to mimic the metabolic rate in vivo (Kuznetsov et al., 1996), the mitochondrial P/O ratio (the amount of ADP administered divided by O_2 consumed) was significantly increased (Larsen et al., 2011). The respiratory control ratio, which is the ratio between state 3 (coupled) and state 4 (uncoupled) respiration, was also significantly increased with $NaNO_3^-$ supplementation (Larsen et al., 2011), as was the maximal rate of ATP production through oxidative phosphorylation. State 2 respiration, indicative of back leakage of protons through the inner mitochondrial membrane, and state 4 respiration were both reduced with $NaNO_3^-$ (Larsen et al., 2011). Therefore, these data indicated that NO_3^- supplementation reduced proton leakage and uncoupled respiration, which increased the mitochondrial P/O ratio. The increased P/O ratio following NO_3^-

supplementation was correlated with the reduction in whole body $\dot{V}O_2$ during exercise (Larsen et al., 2011). Taken together with the findings of Bailey et al. (2010), it appears that NO_3^- supplementation may improve exercise efficiency by improving the efficiency of both muscle contraction (reduced ATP cost of force production) and mitochondrial oxidative phosphorylation (increased P/O ratio).

Implications

It is important to note that dietary or environmental exposure to NO_3^- has historically been considered to be harmful to human health due to a possible increased risk of gastric cancer (Tannenbaum, Weisman, & Fett, 1976) and methemoglobinemia (Comly, 1945/1987). More recent evidence challenges this view and indicates that NO_3^- ingestion (at least through dietary means) may instead confer benefits to human health (reviewed in Bryan & Hord, 2010b; Gilchrist, Winyard, & Benjamin, 2010). Indeed, recent research indicates a role for the NO_3^--NO_2^--NO pathway in improving cardiovascular health (Lundberg et al., 2011), reducing hypertension (Webb et al., 2008), protecting against ischaemia/reperfusion injury (Raat et al., 2009), preventing tissue inflammation (Ohtake et al., 2010), reducing the infarct size in an animal model of myocardial ischaemia (Shiva et al., 2007b), improving brain perfusion (Presley et al., 2011) and inhibiting cancer cell proliferation (Morcos, Carlsson, Weitzberg, Wiklund, & Lundberg, 2010). Therefore, potentiating the NO_3^--NO_2^--NO pathway through adopting a diet rich in NO_3^- (by increasing vegetable consumption) may have far reaching implications for improving human lifelong health. However, further research is required to elucidate the optimal NO_3^- dose and the influence of chronic NO_3^- treatment in various populations on muscle efficiency, fatigue development and exercise performance.

Conclusion

Inorganic NO_3^-, which is abundant in vegetables and is consumed as part of a healthy diet (Bryan & Hord, 2010a), undergoes a stepwise reduction to NO_2^- and NO in an O_2 independent reaction (Figure 1). This pathway of NO synthesis complements the more recognised NOS-NO pathway to preserve NO production when NOS activity is compromised (Bryan et al., 2008; Calström et al., 2010). Recent evidence indicates an important role for this NO_3^--NO_2^--NO pathway in the determination of muscle efficiency and performance (Bailey et al., 2009b, 2010; Kenjale et al., 2011; Lansley et al., 2011a, 2011b; Larsen et al., 2007, 2010, 2011; Vanhatalo et al., 2010; Table I). Mechanistic studies have revealed that NO_3^- supplementation may reduce the ATP cost of muscle force production (Bailey et al., 2010) and increase the efficiency of mitochondrial respiration (Larsen et al., 2011). These findings have implications for enhancing sporting performance in competitive athletes and also for improving functional capacity and quality of life in elderly and/or clinical populations.

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