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Ergogenic effects of dietary nitrate

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Ergogenic effects of dietary nitrate

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on Wednesday 13 February 2019, at 16:00 hours

by

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“To eat is a necessity, but to eat intelligently is an art”

François de la Rochefoucauld

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Chapter 1

General Introduction

Many competitive athletes ingest dietary supplements in addition to their habitual diet with the aim of improving peak exercise performance (1-3). One particular food substance that gained a lot of attention in recent years as a potentially performance-enhancing supplement for endurance and high-intensity type athletes is dietary nitrate. Although promising research findings (4-7) have led to the widespread use of dietary nitrate by athletes, there is currently still limited insight in the various factors that might modulate the effectiveness of dietary nitrate supplementation.

In this thesis, we further explored the potential of dietary nitrate to act as a performance-enhancing nutritional aid by determining whether the beneficial effects can be affected by the duration of supplementation, the nitrate source ingested, and the type of sport performed, while also trying to gain further insight in the *in vivo* pharmacokinetics. In this introductory chapter, we provide an overview of the nitrate-related literature leading up to the studies described in this thesis.

Nitric oxide and the nitrate-nitrite-nitric oxide pathway

Nitrate is a substance that is naturally present in many vegetables and is, as such, readily consumed as part of a normal diet. Research over the past 15 years has shifted from viewing nitrate as a potential harmful substance to regarding nitrate a nutrient that can promote cardiovascular health, as well as a potential performance-enhancing agent (8). While the exact mechanisms behind the ergogenic effects of dietary nitrate are still unknown, it is generally believed that the highly bioactive compound nitric oxide plays a pivotal role in the observed effects. Nitric oxide (NO) is a signaling molecule that serves various functions within different parts of the human body, including skeletal muscle tissue (9). Some of these functions involve the regulation of muscle contractility (10, 11), tissue blood flow (12), mitochondrial oxygen consumption (13), and glucose uptake (**Figure 1.1**) (14). Until very recently, it was believed that NO could only be produced through the oxygen dependent pathway that requires the amino acid L-arginine to be metabolized by nitric oxide synthase (NOS) (15). Indeed, formation of NO through this pathway has been well described in the last decades for the three NOS isoforms, neuronal-NOS (nNOS), inducible NOS (iNOS), and perhaps most extensively, endothelial NOS (eNOS) (16). This is largely due to the fact that the eNOS enzyme is the predominant NOS isoform in (endothelial cells of) blood vessels, and therefore fulfills an import role in vascular health through local production of NO (17).

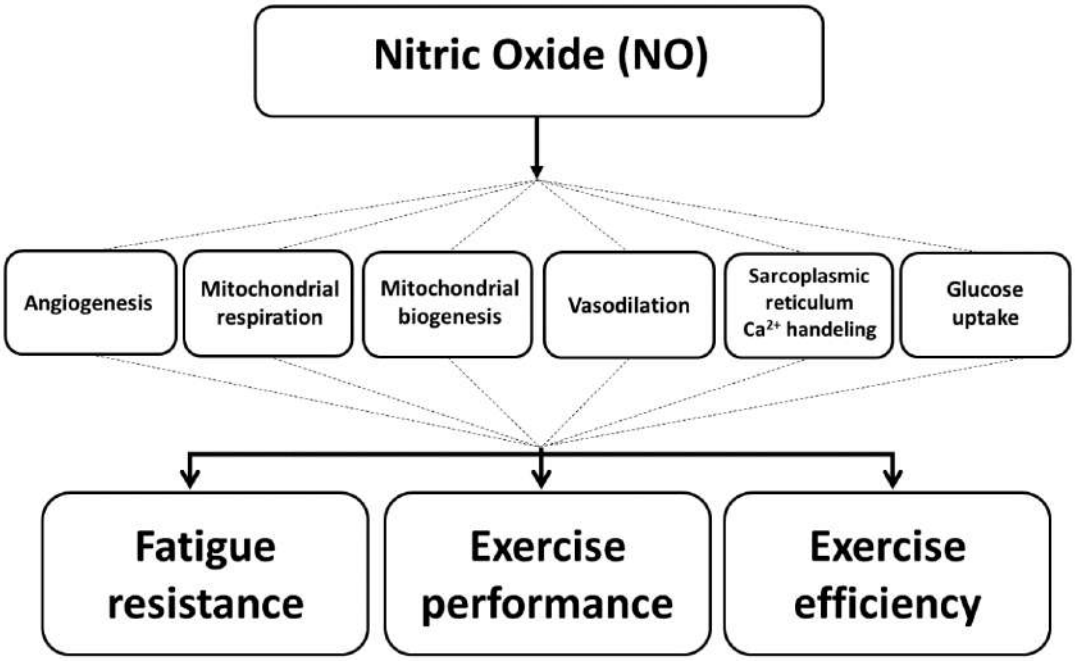

Figure 1.1 Overview of NO related effects believed to be capable of improving exercise performance. Illustration adapted from Jones et al.(18)

In addition to the insights gained on the production of NO through NOS, it was also long accepted that after its formation, NO was subsequently readily oxidized resulting in the formation of inert metabolites known as nitrite and nitrate (19). However, several studies in the mid 1990's speculated on the presence of a reverse pathway that allowed nitrite to be reduced back to NO (20-23). It eventually became apparent that in addition to the oxygen dependent NOS pathway, such a reverse pathway indeed exists, and is predominantly active during acidic and more hypoxic conditions in which NOS activity is hampered (20, 22, 23). The suggestion of greater activity of this pathway in an acidic environment was based on several studies showing associations between higher intragastric NO concentrations with higher salivary nitrite concentrations, as well as NO associated effects, such as increases in gastric mucosal blood flow (20, 23, 24). While at the time, knowledge of the possible *in vivo* complexes capable of reducing nitrite back to NO was far from complete, it was soon suggested that nitrate might also be involved in the proposed reverse pathway (23, 25, 26). The fact that nitrate would first need to be reduced into nitrite was supported by early work, showing that nitrate to nitrite reduction could take place in the human oral cavity (27). Lundberg and Govoni confirmed this hypothesis in their study published in 2004 by showing that *1)* the ingestion of dietary nitrate by human subjects results in an increase in both nitrate and nitrite concentrations in plasma, saliva and urine; and *2)* the increase in nitrite concentrations in plasma can be prevented by giving participants the instruction not to swallow any of the saliva in the first hour after ingesting the dietary nitrate bolus (28). Based on these findings, the authors

suggested that ingestion of dietary nitrate could promote increased NO-bioavailability by the *in vivo* reduction of nitrate to nitrite (primarily in the oral cavity), and the subsequent further reduction of nitrite to NO via various pathways. They described this route as the nitrate-nitrite-NO pathway, which is now widely accepted as an alternative pathway for endogenous NO generation (**Figure 1.2**).

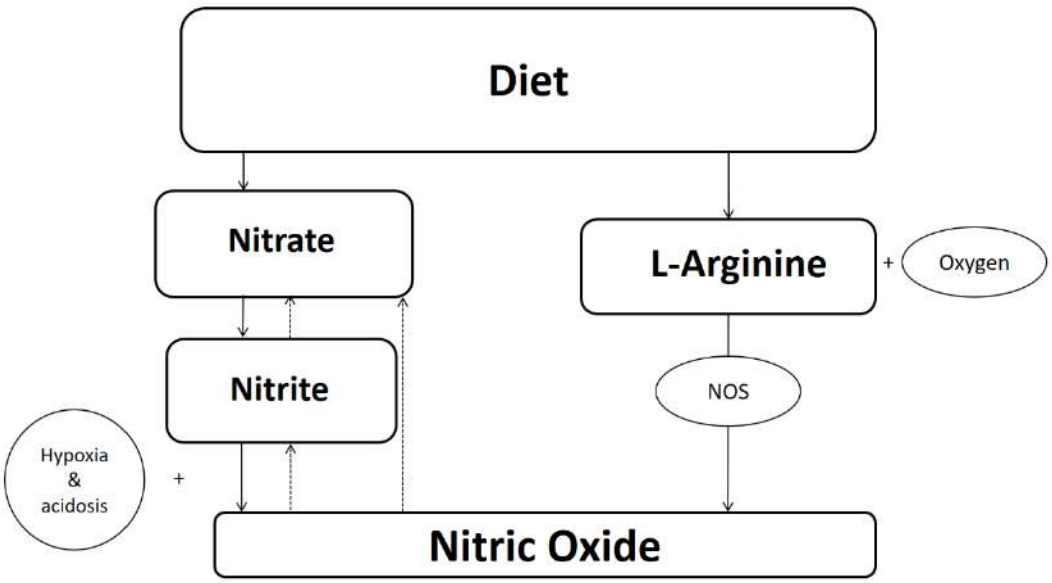

Figure 1.2. The nitrate- nitrite- NO pathway, and the oxygen-dependent NOS mediated pathway.

The same research group followed up on their initial work by assessing whether dietary nitrate could truly serve as an NO donor in humans. In their landmark study for human dietary nitrate research, published as a letter to the editor in the New England Journal of Medicine, Larsen and colleagues showed that 3 days of dietary nitrate ingestion in healthy young volunteers significantly increased plasma nitrate and plasma nitrite concentrations (29). Moreover, this increase in plasma nitrate and nitrite concentrations was accompanied by a 3.7 mmHg decrease in diastolic blood pressure. Although not discussed in that paper, later studies eventually showed that ingestion of nitrate can increase concentrations of the second messenger cGMP, which is a downstream signaling molecule of NO (30-32). An increase in cGMP concentrations has in turn been reported to promote smooth muscle cell relaxation, thereby inducing blood vessel dilation and a decrease in blood pressure (33, 34). The observed effects of dietary nitrate ingestion on blood pressure by Larsen *et al.* thus supported the hypothesis that nitrate was not just an inert metabolite, but that it may serve as a means to increase NO-bioavailability through the so-called nitrate-nitrite-NO pathway. They followed up on these unique findings a year later by assessing whether 3 days of dietary nitrate ingestion would alter physiological and

biochemical parameters during cycling exercise (5). It was hypothesized that nitrate ingestion would change the exercise response in healthy participants as a result of increased NO bioavailability. Again, increases in both plasma nitrate and nitrite concentrations were observed. Remarkably though, the researchers also observed a decrease in oxygen cost during the submaximal cycling exercise, indicative of altered tissue oxygenation. They suggested a decreased proton leakage over the inner mitochondrial membrane to be a likely explanation for their findings. Such decreased proton leakage would allow more ATP to be produced in muscle mitochondria per oxygen molecule consumed (35). The groundbreaking finding of a reduction in oxygen requirement during exercise, as well as the previously observed decrease in blood pressure following the ingestion of dietary nitrate, inspired many researchers in the years that followed to further explore the ergogenic potential, as well as the health benefits of dietary nitrate supplementation. Obviously, this also required more insight to be gained into the various factors that might influence the effectiveness of dietary nitrate ingestion, including the dose and duration of nitrate supplementation, as well as the nitrate source used as an NO donor.

Dietary nitrate supplementation strategy: duration of supplementation

The pioneering studies by Larsen *et al.* (5, 29) have led to a plethora of studies assessing the beneficial effects of dietary nitrate supplementation. Yet, due to the lack of information regarding the dose, duration, and source of dietary nitrate required to elicit ergogenic effects, many different supplementation strategies have been applied. As such, there is currently still no consensus on the optimal protocol for dietary nitrate supplementation. As a first step, Wylie *et al.* provided insight in the minimum dose required to elicit performance effects in healthy recreationally active young participants (36). In their dose-response study, it was shown that a dose of at least ~520 mg of nitrate was required to induce acute changes in exercise parameters (oxygen efficiency and exercise tolerance), when compared to a lower dose of 260 mg nitrate. Interestingly, the beneficial effects that Wylie and colleagues observed occurred after acute ingestion of dietary nitrate, while most of the earlier studies only assessed exercise performance effects following multiple days of dietary nitrate supplementation (4, 5, 37, 38). The fact that the exact mechanism behind the ergogenic effect of dietary nitrate is still unclear has consequently allowed researchers to speculate on the possible benefits of supplementing acutely or for multiple days when aiming to improve exercise performance. Lansley *et al.* (7) for example, showed that an acute dietary nitrate bolus can improve time trial performance in competitive cyclists, and proposed factors such as an increase in muscle blood flow due to vasodilation, to serve as a possible explanation for their findings.

Alternatively, Cermak *et al*. (39) showed that 6 days of dietary nitrate ingestion improved time trial performance, and decreased submaximal oxygen cost in moderately trained cyclists. The authors speculated on whether adaptations such as reduced "slippage" of the mitochondrial proton pump, as suggested by Larsen *et al.* (40), may be responsible for the observed ergogenic effects. Using a multiday supplementation approach would then seem just, as adaptations in mitochondrial efficiency are believed to unlikely occur following acute dietary nitrate ingestion (40). This however remains speculation as a clear comparison between the effects of acute versus multiday dietary nitrate supplementation protocols on exercise performance is lacking. The current notion that (acute) performance effects following dietary nitrate ingestion only occur in recreationally and moderately trained endurance athletes further fuels debate on whether more well trained endurance athletes might require multiday dietary nitrate supplementation regimens to boost performance (41, 42).

Dietary nitrate supplementation strategy: nitrate source

For many years, nitrate was primarily known in the form of nitrate glyceryl tri-nitrate, an organic nitrate used in the medical field to relieve discomfort when a patient experiences an angina attack. However, research into the nitrate-nitrite-NO pathway has shifted attention towards dietary nitrate also serving as an NO precursor (43). Dietary nitrate is an inorganic nitrate that is chemically different from the organic nitrate used for medicinal purposes. While organic nitrate is industrially synthesized through a reaction between nitric acid and an alcohol group, inorganic nitrate as found in vegetables can be naturally formed, primarily through the bacteria-mediated bonding of atmospheric nitrogen and oxygen, and a metal cation, mostly sodium or potassium (43). This natural bond is highly soluble in water and therefore ends up in the soil and gets taken up by plants, especially beetroot and green leafy vegetables. Alternatively, it is also possible to industrially produce inorganic sodium nitrate and potassium nitrate, which fulfill a variety of functions, including being food preservatives for cured meats. In the early work by Lundberg and colleagues, sodium nitrate was administered orally to participants (28). Using sodium chloride as a taste matched placebo, they were able to assess the effects of sodium nitrate in a randomized, placebo controlled fashion. As the use of sodium nitrate was strictly regulated in certain countries, researchers aiming to follow up on this work resorted to an alternative source, which was the naturally nitrate-rich root vegetable called red beetroot (*beta vulgaris*) (4, 44). Beetroot was already being processed into a beverage for commercial purposes, making it a readily available food product with no legal limitations. Moreover, the nitrate content could be kept within a certain range, providing researchers with a useful nitrate source. The first study with beetroot juice published in

2008 confirmed the findings by Larsen *et al.* (5), by showing that ingestion of nitrate-rich beetroot juice increases plasma nitrate and nitrite concentrations, and decreases blood pressure in healthy young subjects (44). Furthermore, the study extended on those earlier findings by also showing that dietary nitrate could reduce platelet activation, which is an effect suggested to result from increased NO-bioavailability (45). More importantly, this work showed that the beneficial effects of dietary nitrate could be induced with different nitrate sources, including naturally nitrate-rich food sources. Since then, several studies have indeed shown that nitrate-rich vegetables, such as spinach and lettuce, can increase plasma nitrate and nitrite concentrations (46), and decrease blood pressure (47). However, to what extent the pharmacokinetic response and associated physiological or functional responses may differ when various nitrate sources are compared, is not yet clear.

Dietary nitrate supplementation strategy: the type of sports

The remarkable finding of reduced oxygen cost during submaximal exercise following nitrate ingestion (5, 38, 40), inspired many researchers to focus on the effects of dietary nitrate supplementation on endurance-type exercise performance. Apart from the improved exercise efficiency, improvements were observed in terms of increased time to exhaustion, and more importantly, actual performance measures such as cycling and running time trials. Interestingly though, improvements in performance only seemed to occur in recreational and moderately trained endurance athletes (6, 7), while ergogenic effects remained absent in highly trained and elite endurance athletes (48). The optimized physiological adaptations to years of endurance training in the endurance athletes, as well as the primarily oxidative production of ATP, have since been proposed as factors that may attenuate the formation of an acidic and hypoxic environment that can facilitate nitrate to NO reduction (49, 50). Furthermore, rodent studies (51, 52) have shown that dietary nitrate might especially be effective in improving muscle contraction and blood flow of muscles consisting of primarily type II fibers. Collectively, these findings suggest that dietary nitrate may show greater performance improvements in high intensity-type sports. A growing number of studies are therefore currently assessing the effectiveness of dietary nitrate supplementation during these more high intensity-type exercise activities, and recent observations even indicate possible exercise performance benefits in highly trained, and elite athletes (53).

Muscle as a nitrate buffer

With NO being a volatile compound with a very short half-life in the human body, it is extremely difficult to assess the *in vivo* bio-availability of NO. Therefore, it has become common practice to determine the pharmacokinetics of dietary nitrate by measuring plasma nitrate and plasma nitrite concentrations. Indeed, most studies have been limited to reporting plasma nitrate and nitrite concentrations in relation to subsequent performance or (cardiovascular) health effects. However, recent findings in rodents suggest that nitrate and nitrite, at least in the basal state, are present in different bodily tissues (54). In their study, Piknova *et al*. (54) compared the nitrate and nitrite content of blood, liver and skeletal muscle tissue in wild type mice and rats. In line with previous findings, they observed that nitrate (and nitrite) was indeed present in blood and liver tissue. Importantly though, they also provided the first evidence of nitrate being present in skeletal muscle tissue, and showed that nitrate concentrations in muscle tissue in the basal state were far greater than blood and liver concentrations (**Figure 1.3**).

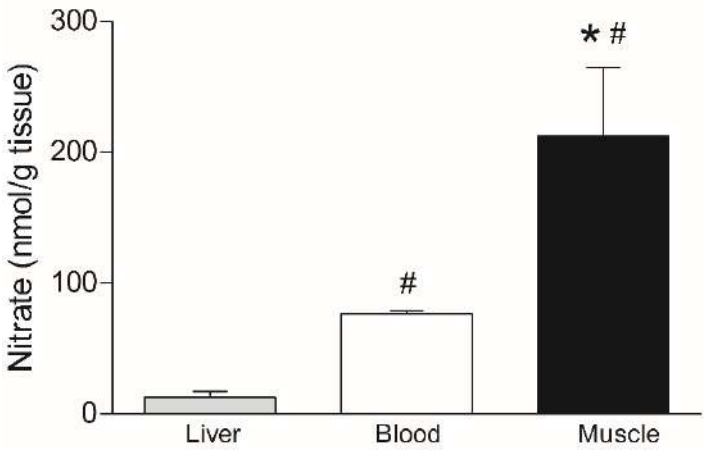

Figure 1.3. Data of basal nitrate concentrations in liver, blood and muscle in adult male Wistar rats (n=6). # Significantly different when compared with liver. * Significantly different when compared with blood. Illustration adapted from Piknova *et al.* (54).

Based on these differences in nitrate concentrations, it was suggested that skeletal muscle tissue might act as an endogenous nitrate buffer. The nitrate stored in skeletal muscle tissue could potentially serve as a stable storage pool for local NO production via endogenous reduction through the nitrate-nitrite-NO pathway. Although it remains to be determined whether this actually occurs and whether a similar storage of nitrate exists in human skeletal muscle tissue, these findings do raise the question to what extent it is appropriate to limit assessment of the pharmacokinetics of dietary nitrate to the measurement of plasma concentrations. Taking into account that some of the reported

improvements in exercise performance following nitrate ingestion have been suggested to result from adaptations at the muscle level (51, 52, 55, 56), it appears essential to look beyond what is known at the systemic level, and to study nitrate content of other tissues in the human body. This should not only include assessment of nitrate concentrations in the basal state, but should also address whether such local storage may be affected by dietary nitrate intake.

Hypoxia and NO

In contrast to the gap in knowledge regarding the presence of nitrate and nitrite in other tissues besides the systemic circulation, a substantial body of research has been dedicated to defining factors that can facilitate reduction of these anions to NO. *In vitro* work has shown that an environment that is acidic and low in oxygen can strongly potentiate the activity of the nitrate-nitrite-NO pathway (20). This may, in part, explain why it is currently believed that dietary nitrate preferentially exerts beneficial effects on fast-twitch muscle fibers (49). As previously described, rodent studies have shown a fiber-type specific effect of nitrate supplementation, with increased muscle contractility and blood flow primarily being observed in muscle tissue containing more type II muscle fibers (51, 52). While the mechanisms behind this effect remain unclear, it has been proposed that the lower intramyocellular oxygen pressure observed during contractions of fast-twitch muscle fibers may allow greater nitrite to NO reduction (52). Several nitrate related studies performed in humans embraced this hypothesis and assessed exercise performance in different low oxygen conditions. The first study that provided insight in this area was from Kenjale *et al.* in 2011 (57). They assessed whether dietary nitrate could improve exercise performance in peripheral arterial disease patients, as this is a disease characterized by an impaired blood flow and a reduced oxygen supply to the (lower) extremities. Compared to a placebo, acute ingestion of nitrate rich beetroot juice increased the walking time of the subjects by 18%, before the onset of claudication pain. Vanhatalo *et al.* soon after showed that dietary nitrate ingestion improves knee-extension exercise performance in healthy volunteers subjected to systemic normobaric hypoxia (58). Dietary nitrate ingestion reduced PCr degradation and Pi accumulation in the exercising muscle in hypoxia, when compared to a placebo. Furthermore, nitrate restored high intensity knee-extension exercise tolerance to what was observed in the normoxic control situation, while placebo ingestion resulted in a decreased performance. These findings were later supported by Masschelein *et al*. (59), who also found that dietary nitrate supplementation was effective in negating the decrease in cycling exercise tolerance during systemic hypoxia. Furthermore, ingestion of the nitrate-rich supplement was shown to decrease systemic oxygen uptake, and local oxygen cost of submaximal exercise, as assessed by

NIRS (59). Overall, these studies indicate a clear effect of how activation of the nitrate-nitrite-NO pathway can attenuate the detrimental effect of (systemic) hypoxia on exercise performance, perhaps by increasing NO-bioavailability when the NOS pathway is impaired.

Blood Flow Restriction and NO

Whereas the previously discussed studies assessed the effects of dietary nitrate ingestion on exercise performance during *systemic* hypoxia (58-61), Hoon *et al*. recently examined whether nitrate supplementation could reduce muscular fatigue of the knee extensors during *local* hypoxia (62). In line with their hypothesis, they observed that nitrate ingestion increased exercise tolerance during exhaustive quadriceps contractions when blood flow was restricted. The blood flow restriction procedure (BFR) used in the study involved a cuff, similar to a blood pressure cuff but slightly larger in size, being placed proximally on the upper leg. The authors speculated that the local hypoxic environment created with BFR may have allowed nitrate to improve fast-twitch muscle fiber function, and/or increase NO-mediated perfusion to the exercising muscle. They based this explanation on the previously discussed rodent studies by Hernandez *et al.* (51) and Ferguson *et al.* (52), showing increased muscle contractility and blood flow of primarily fast-twitch fiber containing muscles following nitrate ingestion.

While Hoon *et al.* only applied the BFR procedure to assess the effects of dietary nitrate on exercise tolerance during local hypoxia, there is an increasing amount of evidence suggesting that exercising with BFR can also stimulate muscle hypertrophy. Although low volume, low load resistance-type exercise is considered ineffective in stimulating muscle hypertrophy, transient restriction of blood flow towards and from the exercising muscle has recently been shown to acutely stimulate muscle protein synthesis (63-66), and result in skeletal muscle hypertrophy when applied as a prolonged resistance-type exercise training (67, 68). Despite the exact mechanisms still being unclear, the transient local ischemia and hypoxia, and subsequent hyperemic reperfusion have been proposed as factors that can set off certain anabolic triggers (66, 69, 70). Interestingly, hypoxia has also been reported to strongly stimulate nitrite to NO reduction (Figure 1.2). As such, it is tempting to speculate on a potential role for NO in the anabolic effect of BFR. Although it was recently shown that nitrate supplementation by itself does not augment the muscle protein synthetic response to protein ingestion in type 2 diabetes patients (71), further insight into the conditions under which local hypoxia/ischemia may induce anabolic events could open up future paths for further elaborating on the beneficial effects of increased NO production through the nitrate-nitrite-NO pathway.

Outline of this thesis

The studies included in this thesis were focused on assessing factors that may modulate the ergogenic effect of dietary nitrate supplementation. First, we evaluated whether certain characteristics of nitrate supplementation protocols would be associated with different plasma nitrate/nitrite concentrations and/or functional effects. In **Chapter 2** we questioned whether the duration of dietary nitrate supplementation (acute vs 6 days) affects cycling performance in highly trained endurance athletes. In **Chapter 3,** we show that different nitrate sources have fairly similar effects on plasma nitrate and nitrite concentrations, but that the blood pressure lowering effects appear to be more pronounced with 'natural nitrate sources' such as beetroot, rocket salad and spinach, when compared to sodium nitrate. In **Chapter 4**, we assessed whether dietary nitrate supplementation could also be effective in improving repeated high-intensity running performance in well trained soccer players. In the remainder of this thesis, we aimed to further explore the possibility of the nitrate-nitrite-NO pathway mediating local effects in skeletal muscle tissue. In **Chapter 5**, we first followed up on data from animal studies and determined whether the pharmacokinetics of dietary nitrate extend beyond the observed changes in plasma concentrations. We therefore assessed the basal nitrate content of skeletal muscle tissue *in vivo* in humans, as well as the possible changes in this content following dietary nitrate ingestion. In **Chapter 6** we describe whether applying local transient hypoxia/ischemia to a muscle through the blood flow restriction procedure, with and without exercise, affects muscle protein synthesis rates in healthy young men. Finally, in the general discussion (**Chapter 7**), we further discuss the possibility of skeletal muscle tissue serving as a nitrate reservoir, taking in account the findings described in this thesis as well as supporting evidence from the literature. Additionally, future research opportunities for nitrate are identified that could further elucidate on the existence and role of a skeletal muscle nitrate buffer.

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Chapter 2

No effect of acute and 6-day nitrate supplementation on VO_2 and time-trial performance in highly trained cyclists

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Abstract

Background: While the majority of studies reporting ergogenic effects of dietary nitrate have used a multiday supplementation protocol, some studies suggest that a single dose of dietary nitrate prior to exercise can also improve subsequent performance. We aimed to compare the impact of acute and 6-day sodium nitrate supplementation on oxygen uptake ($\dot{V}O_2$) and time-trial performance in trained cyclists.

Methods: Using a randomized, double blind, cross-over design, 17 male cyclists (25±4 y, $\dot{V}O_{2peak}$ 65±4 mL·kg^{-1}·min^{-1}, W_{max} 411±35 W) were subjected to 3 different trials; 5 days placebo and 1 day sodium nitrate supplementation (1-DAY); 6 days sodium nitrate supplementation (6-DAY); 6 days placebo supplementation (PLA). Nitrate was administered as 1097 mg sodium nitrate providing 800 mg (~12.9 mmol) nitrate per day. Three hours after ingestion of the last supplemental bolus, indirect calorimetry was performed while subjects performed 30 min of exercise at 45% W_{max} and 30 min at 65% W_{max} on a cycle ergometer, followed by a 10 km time-trial.

Results: Immediately prior to exercise, plasma [nitrate] and [nitrite] increased to a similar extent during the 6-DAY and 1-DAY trial, but not with PLA (plasma nitrite: 501±205, 553±278, and 239±74 nM, respectively; *P*<0.001). No differences were observed between interventions in $\dot{V}O_2$ during submaximal exercise, or in time to complete the time-trial (6-DAY: 1004±61, 1-DAY: 1022±72, PLA: 1017±71 s; *P*=0.28).

Conclusion: We conclude that both acute and 6-days of sodium nitrate supplementation do not alter $\dot{V}O_2$ during submaximal exercise or improve time-trial performance in highly-trained cyclists, despite increasing plasma [nitrate] and [nitrite].

Introduction

An increasing body of evidence suggests that inorganic nitrate supplementation can have beneficial effects on exercise performance (1-5). Previous studies reported reduced oxygen uptake ($\dot{V}O_2$) during submaximal exercise (1-3), enhanced exercise tolerance (1, 6), and improved time-trial performance (2, 5) following ingestion of dietary nitrate in humans. A reduction of the ingested nitrate by facultative bacteria in the oral cavity into nitrite can eventually lead to an increased bio-availability of nitric oxide (NO) (7). This increased NO bio-availability has been associated with reduced $\dot{V}O_2$ during submaximal exercise and increased blood flow during exercise (8, 9).

Larsen *et al.* (3) were the first to study the effects of dietary nitrate supplementation on $\dot{V}O_2$ during exercise. They observed a lower $\dot{V}O_2$ during submaximal cycling exercise following 3 days of sodium nitrate ingestion. These findings were later confirmed in studies that used beetroot juice as the nitrate source (1, 2). Interestingly, the ingestion of nitrate-rich beetroot juice for 6 consecutive days did not only result in a reduced $\dot{V}O_2$ during submaximal cycling exercise, but also increased time to exhaustion during constant work high-intensity cycling (1). In an attempt to determine whether the observed effects translate to functional performance benefits, we previously assessed the effect of nitrate on time-trial performance (2). We showed that 6 days of beetroot juice supplementation resulted in a 1.2% improvement in cycling time-trial performance compared to a nitrate-depleted placebo.

Whereas most of the earlier studies used a multi-day (≥3 days) supplementation protocol to elicit the ergogenic effects of dietary nitrate (1, 2), other studies adopted a so-called 'acute' supplementation regimen (10, 11). Vanhatalo *et al.* (11) reported reduced $\dot{V}O_2$ during submaximal exercise following the acute (i.e. 2.5 h prior to exercise) ingestion of beetroot juice. This effect persisted when nitrate supplementation was continued for 5 and 15 days. Likewise, acute nitrate supplementation was observed to improve functional performance during a simulated 4- and 16-km cycling time-trial (5). These observations suggest that a single nitrate dose might be as effective in improving performance as a multi-day supplementation protocol. However, multi-day nitrate supplementation has been shown to induce beneficial structural adaptations at the myocellular level which are unlikely to manifest within 1-3 h after ingestion of a single nitrate bolus (8, 12). As such, a multiday regimen might be necessary to obtain the full ergogenic benefits of nitrate supplementation. In accordance, improvements in exercise performance after acute nitrate supplementation have been observed by some (5, 6), but certainly not all studies (10, 13-15). Interestingly, recent work also suggests minimal to no performance enhancing effects of dietary nitrate in highly-trained/elite athletes, with most of the studies adopting

an acute supplementation protocol (10, 16-18). Since there are no studies that have comprehensively compared the effects of acute and chronic nitrate supplementation in trained cyclists, it is currently unknown whether a longer supplementation protocol would be more beneficial than ingestion of a single dose of dietary nitrate. Therefore, the aim of the present study was to determine the effect of acute and 6-day dietary nitrate supplementation on $\dot{V}O_2$ during submaximal exercise, and time-trial performance in highly-trained cyclists. We hypothesized that 6 days of nitrate ingestion would lower $\dot{V}O_2$ during submaximal exercise and improve time trial performance.

Methods

Subjects

Twenty male competitive cyclists/triathletes participated in this study that was approved by the medical ethical committee of the Maastricht University Medical Centre, the Netherlands. After explanation of the protocol, all subjects provided written informed consent. Three subjects failed to complete the study because of injury (*n*=1), or personal time constraints (*n*=2). Subjects' characteristics of the remaining 17 subjects are provided in **Table 2.1**.

Table 2.1. Subjects' characteristics

Age (y)	25 ± 4
BMI (kg/m^2)	21.8 ± 1.8
Cycling experience (y)	9.6 ± 5.1
Cycling h/wk	9.7 ± 3.7
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	65 ± 4
W_{max} (W)	411 ± 35

All values are means ± SD (n=17).

Study design

In this double-blind, randomized, placebo controlled cross-over study, subjects engaged in 3 experimental test-days to study the effects of acute and 6-day supplementation of a dietary sodium nitrate solution ($NaNO_3$) in comparison to a placebo (NaCl). On each test-day, $\dot{V}O_2$ during submaximal cycling, and time-trial performance were assessed. After screening (visit 1), subjects visited the laboratory ~1 week prior to the first trial for familiarization of the full protocol, including 2x 30 min submaximal cycling exercise and the 10-km time-trial (visit 2). Subsequently, the three test-days (visits 3-5) were each preceded by the subjects consuming a supplemental bolus for 5 consecutive days. The last (6th) bolus was consumed in the laboratory on the test-day, 3 h prior to the exercise tests. The supplementation periods were interspaced by 8 days of wash-out.

Pre-testing

Baseline characteristics were determined during screening (Table 2.1). Subjects' peak power output (W_{max}) and peak oxygen consumption ($\dot{V}O_{2peak}$) were determined while performing a stepwise exercise test to exhaustion on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), using an online gas-collection system (Omnical, Maastricht University, The Netherlands). After a 5-min warm-up at 150 W, the workload was increased by 50 W every 2.5 min until exhaustion. $\dot{V}O_{2peak}$ was defined as the median of the highest consecutive $\dot{V}O_2$ values over 30 s. W_{max} was calculated as follows: $W_{max}=W_{completed}+t_{uncompleted}/150\ (s)\times 50$ (19). The $W_{completed}$ was the power output at the last completed step, and the $t_{uncompleted}$ was the time (s) spent in the last uncompleted step.

Supplementation protocol

In the five days leading up to the test-days (visits 3-5), subjects either consumed a daily dose of sodium nitrate ($NaNO_3$ [BASF, Ludwigshafen, Germany]) or an equal amount of NaCl (Frisia Zout BV, Harlingen, The Netherlands) dissolved in 140 mL of water after breakfast. The 6-day nitrate supplementation period (6-DAY) consisted of 6 days of 1097 mg $NaNO_3$ ingestion, providing 800 mg (~12.9 mmol) nitrate/day. As the placebo intervention (PLA), subjects ingested 1097 mg/day NaCl for 6 days. The acute nitrate trial (1-DAY) consisted of a 1097 mg $NaNO_3$ bolus that was ingested on the test-day (day 6), preceded by 5 days of NaCl ingestion.

Experimental test-days

On the morning of the final (6^{th}) day of each supplemental period, subjects reported to the laboratory after an overnight fast. Following a 10-min rest period, blood pressure was measured with subjects in the supine position using an automated blood pressure monitor (Omron Healthcare Inc, Field Court Lake Forest, USA), and a catheter was inserted into an antecubital vein. After obtaining a fasted blood sample, subjects consumed a standardized breakfast, immediately followed by their last (6^{th}) supplemental beverage. During the subsequent 3-h rest period, repeated blood draws were performed at 90, 150, 180, 240, and 270 min and a second blood pressure measurement was performed 2.5 h post-ingestion of the last supplement. After the 3-h rest period, subjects performed a 1-h submaximal exercise test on a cycle ergometer at 45% W_{max} (30 min) and 65% W_{max} (30 min). Measurements of $\dot{V}O_2$ and CO_2 production were obtained through the use of a respiratory facemask, connected to a gas-collection system (2). Respiratory data and heart-rate (HR) (Polar, Finland) were collected continuously for 5–7 min at 5, 20, 35, and 50 min into the submaximal exercise test, and the last 3-min were averaged. Following the submaximal test, subjects performed a simulated ~10-km cycling time-trial. The

amount of work to be performed was calculated as follows: *total amount of work (J) = 0.85*W_{max}*900 (s)* (20). The ergometer was set in linear-mode so that 85% W_{max} was achieved when subjects cycled at their preferred pedaling rate of 85±7 rpm, as determined during familiarization. Subjects received no verbal or physiological feedback during the time-trial, and were only aware of the absolute (kJ) and relative (%) amount of work performed. Ratings of perceived exertion (RPE) were assessed after each 30-min submaximal exercise test and after the time-trial using the Borg 6–20 scale (21). All testing was performed under standardized conditions (18.3±0.1 °C, 58±1% humidity) on the same time of day, and the same day in the week.

Physical activity and dietary standardization

Subjects recorded their dietary intake and physical activity for three days prior to the first test-day (visit 3), and refrained from strenuous activity for 48 h prior to the test. Subjects replicated their dietary intake and activities in the days prior to the following two test-days. Subjects did not consume caffeine (12 h) or alcohol (24 h) prior to each visit. To prevent attenuation in the reduction of nitrate to nitrite, subjects refrained from using antibacterial mouthwash/toothpaste during the 6-day supplementation period (22). No restrictions were set for the intake of nitrate-rich foods. However, subjects were provided with a standardized dinner for the evening before (~41 mg nitrate), and a standardized breakfast (~7 mg nitrate) on each test-day, which was adapted to their bodyweight as described previously (2). The *ad libitum* amount of water consumed during the first test-day was replicated during the second and third test-day.

Plasma analysis

Blood samples were collected in S-Monovette® Lithium-Heparin containing tubes (Sarstedt, Nümbrecht, Germany) and immediately centrifuged at 1000 *g* for 5 min, at 4 °C. Aliquots of plasma were frozen in liquid nitrogen , and stored at –80 °C for subsequent analysis of plasma [nitrate] and [nitrite] using chemiluminescence, as described previously (13).

Statistical analysis

Performance data from the time-trials as well as $\dot{V}O_2$ data were analyzed by one-way repeated measures ANOVA with treatment (PLA vs 1-DAY vs 6-DAY) as factor. Statistical analysis of all plasma data was performed using two-way repeated measures ANOVA with time and treatment as within subject factors. Statistical significance was set at $P<0.05$, and any interaction or main effect was subsequently analyzed using Bonferroni post-hoc test. All data were analyzed using SPSS 21.0 (SPSS Inc, USA), and are presented as means ± SD.

Results

Plasma [nitrate] and [nitrite]

Plasma nitrate concentrations at baseline (*t*=0) were higher in the 6-DAY trial when compared with 1-DAY and PLA trials (160±53 vs 74±26 and 71±16 µM, respectively; *P*<0.001). Following ingestion of the final bolus, plasma [nitrate] increased to the same extent in the 6-DAY (+316±136 µM) and 1-DAY trial (+360±150 µM) and remained elevated throughout the exercise protocol (t=180 min and beyond), while no changes were observed during the PLA trial (**Figure 2.1A**). In contrast to plasma [nitrate], plasma [nitrite] was not different between trials at baseline (**Figure 2.1B**). Following ingestion of the final bolus, plasma [nitrite] increased ~2-fold prior to exercise (t=180 min) during the 6-DAY (from 263±104 to 501±206 nM; *P*<0.001) and the 1-DAY trial (from 216±81 to 553±278 nM; *P*<0.001), whereas no changes were observed during the PLA trial (from 229±76 to 239±74 nM; *P*=0.49; Figure 2.1B). Plasma [nitrite] did not differ between the 1-DAY and 6-DAY trial at any time point.

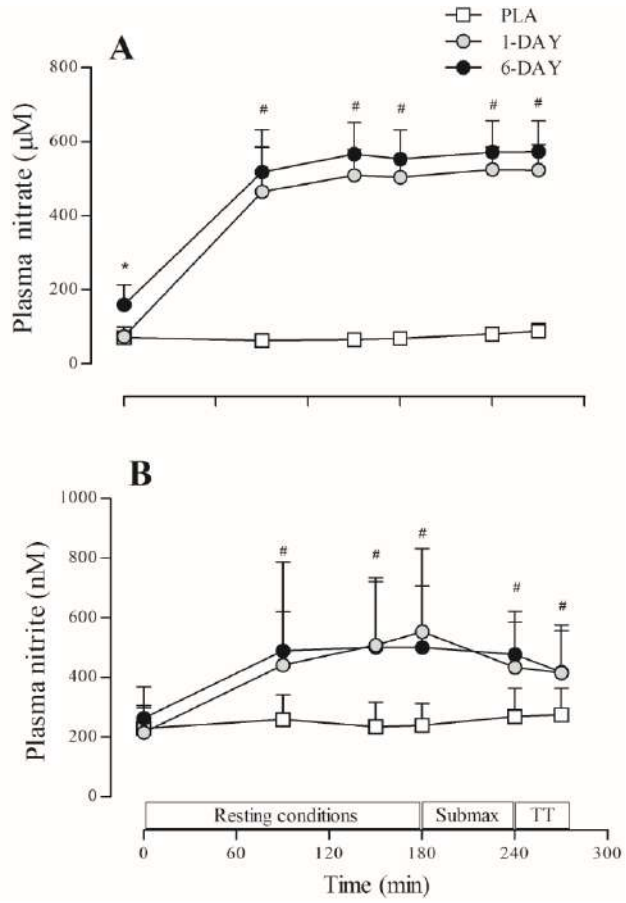

Figure 2.1. Mean plasma nitrate (A) and nitrite (B) concentrations on trial days during resting conditions and during the submaximal cycling test (Submax) and the 10-km time-trial (TT) for the placebo (PLA), the acute nitrate (1-DAY) and the 6-day nitrate (6-DAY) intervention. *6-DAY significantly different from 1-DAY and PLA. #1-DAY and 6-DAY significantly different from t=0 min (within treatment) and different from PLA (between treatments). Data are means ± SD (n= 17)

Submaximal cycling exercise

Mean $\dot{V}O_2$ was not different between the 6-DAY, 1-DAY and PLA trial while cycling at 45% W_{max} (*P*=0.60; **Figure 2.2**). After adjusting the workload to 65% W_{max}, $\dot{V}O_2$ increased in all 3 trials, but no differences were observed in mean $\dot{V}O_2$ between trials (*P*=0.89). Similarly, no differences were observed between trials for RER (*data not shown*), average HR and RPE during both the 45% W_{max}, and 65% W_{max} workload (**Table 2.2**; all *P*>0.20).

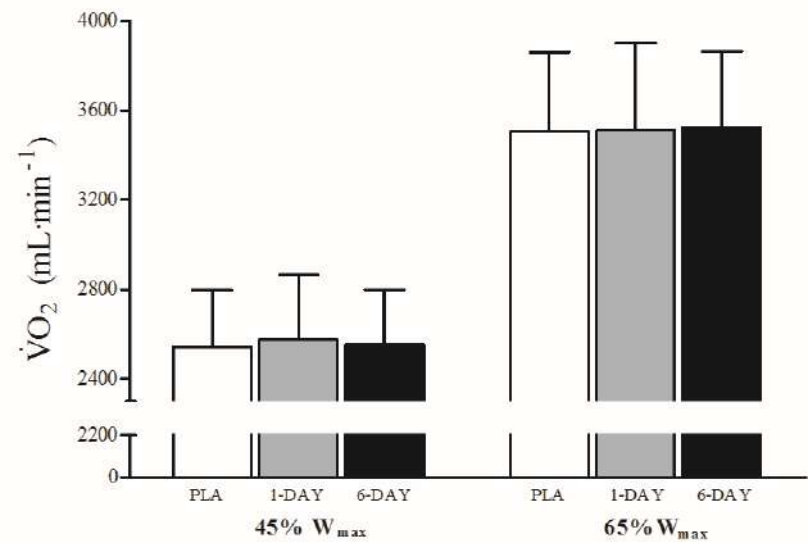

Figure 2.2. Mean oxygen uptake (VO_2) during submaximal cycling at 45% and 65% of maximal power output (W_{max}) for the placebo (PLA), the acute nitrate (1-DAY) and the 6-day nitrate (6-DAY) trial. No differences were observed between experimental trials. Data are means ± SD (n = 17).

TABLE 2.2. Blood pressure and heart rate data

	PLA	**1-DAY**	**6-DAY**
Blood pressure SYS (t=0h) (mmHg)	121 ± 8	122 ± 5	120 ± 8
Blood pressure SYS (t=2.5h) (mmHg)	121 ± 6	122 ± 7	122 ± 9
Blood pressure DIA (t=0h) (mmHg)	65 ± 7	64 ± 7	64 ± 8
Blood pressure DIA (t=2.5h) (mmHg)	61 ± 9	62 ± 7	61 ± 6
Mean Heart rate 45% W_{max} (beats/min)	133 ± 10	134 ± 11	131 ± 9
Mean Heart rate 65% W_{max} (beats/min)	166 ± 13	168 ± 10	166 ± 10
Mean Heart rate TT (beats/min)	178 ± 6	177 ± 6	175 ± 7
Peak Heart rate TT (beats/min)	187 ± 7	187 ± 7	183 ± 8[1]
RPE after 45% W_{max}	11 ± 1	11 ± 1	11 ± 1
RPE after 65% W_{max}	16 ± 2	16 ± 2	16 ± 2
RPE after TT	19 ± 1	19 ± 1	19 ± 1

All values are means ± SD (n=17). Placebo (PLA), acute nitrate (1-DAY), 6-day nitrate (6-DAY), systolic (SYS), diastolic (DIA), peak power output (W_{max}), time-trial (TT) and Rate of perceived exertion (RPE). No differences over time or between interventions were observed for blood pressure. There was a main effect for peak heart rate, which tended to be lower for 6-DAY vs 1-DAY ([1]*P*=0.10).

Time Trial performance

The average time to complete the 10-km time-trial was 1004±61 s (6-DAY), 1022±72 s (1-DAY), and 1017±71 s (PLA), with no apparent differences between the interventions (*P*=0.28; **Figure 2.3A**). In accordance, no differences were observed in average power output during the time-trial between the 3 interventions (*P*=0.33; **Figure 2.3B**), nor for the power output at every 10% stage of the time trial (*P*=0.54, *data not shown)*. For both RPE and HR, no differences were observed between interventions (**Table 2.2**).

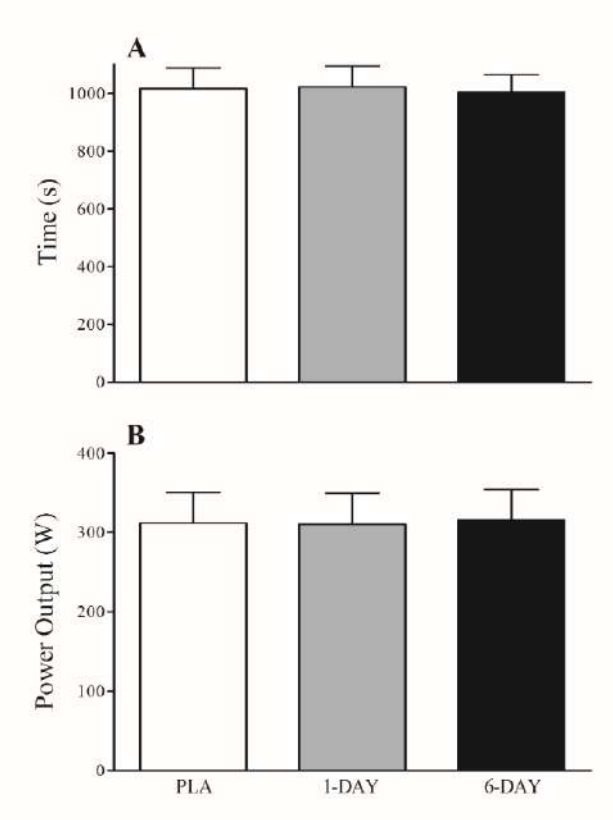

Figure 2.3. Time to complete the time trial (A) and mean power output (B) during the ~ 10-km time trial for the placebo (PLA), the acute nitrate (1-DAY) and the 6-day nitrate (6-DAY) trial. No differences were observed between experimental trials. Data are means ± SD (n = 17).

Discussion

In the present study, we demonstrated that nitrate supplementation with an acute or 6-day supplementation protocol resulted in similar increases in plasma [nitrate] and [nitrite]. However, acute as well as 6-day dietary nitrate supplementation did not alter $\dot{V}O_2$ during submaximal exercise or improve time-trial performance in highly-trained cyclists.

Nitrate-rich supplements have become increasingly popular among both elite and recreational athletes over the past years. Following the first studies reporting reduced $\dot{V}O_2$

during submaximal exercise and improved exercise performance (1-3, 5), a plethora of different supplementation regimens have been used to explore the ergogenic effects of nitrate. Whereas the majority of studies in this area showed ergogenic effects following multiple days of nitrate supplementation, some suggest that ingestion of a single dose of nitrate prior to exercise can also improve performance (5, 6). However, fundamental work exploring the underlying mechanisms of nitrate's ergogenic properties has shown that improved mitochondrial efficiency and contractile function following chronic nitrate supplementation are associated with reduced expression of ANT (8) and increased expression of calcium-handling proteins (12). Such changes are unlikely to occur after the acute ingestion of dietary nitrate. Hence, we speculated that greater benefits can be expected from multi-day supplementation than from acute supplementation. The present study is the first to assess the effects of both acute and 6-days of nitrate supplementation on $\dot{V}O_2$ and time-trial performance within the same group of highly-trained cyclists. We found that supplementation with 800 mg nitrate, provided both acutely and successively for 6 days, resulted in a ~6-fold increase in plasma [nitrate] and a ~2-fold increase in plasma [nitrite], in comparison to a placebo (Figure 2.1). Interestingly, in line with previous work by Vanhatalo *et al*. (11), the elevated plasma [nitrate] and [nitrite] in the hours following ingestion of the final bolus did not differ between acute and 6-day nitrate supplementation. Therefore, potential differences in ergogenic effects between acute and prolonged nitrate supplementation would more likely be attributed to an adaptive response to nitrate availability, rather than circulating (peak) [nitrate] or [nitrite].

Despite the substantially elevated plasma [nitrate] and [nitrite] after acute and 6-days of nitrate supplementation, we did not observe any ergogenic or pharmacodynamic effects in the highly-trained cyclists in the present study. Given the time required for structural myocellular adaptations to occur (8), we expected the 6-DAY protocol to be more beneficial than the 1-DAY protocol. In contrast to our hypothesis, $\dot{V}O_2$ during submaximal cycling did not differ between interventions, and no differences were observed in time-trial performance (Figures 2.2 and 2.3). These findings do not match our previous observations of ergogenic effects following 6-day supplementation in moderately trained athletes with a $\dot{V}O_{2peak}$ of 58 $mL \cdot kg^{-1} \cdot min^{-1}$ (2). However, the lack of ergogenic effects observed in the present study with better trained subjects ($\dot{V}O_{2peak}$ ~65 $mL \cdot kg^{-1} \cdot min^{-1}$) is in line with recent literature on highly-trained and elite athletes (14, 17, 23, 24). Indeed, $\dot{V}O_2$ and time-trial performance remained unchanged following nitrate supplementation in highly-trained cross-country skiers (17) and middle-distance runners (14). Although the exact reason for the impaired response to nitrate in trained individuals is still unclear, some viable explanations have emerged in recent literature. For example, two animal studies reported that nitrate preferably exerts its effects through type II muscle fibers (9,

12). Endurance-trained athletes, such as those included in the current study, likely have a low proportion of these fibers (25), possibly limiting their ability to fully benefit from the effects of nitrate supplementation. In addition, previous observations suggest that regular (endurance-type) exercise results in structural adaptations in various tissues, most likely in response to elevated NOS activity (26). These physiological changes are believed to account for the attenuated response to elevations in NO-bioavailability in elite athletes (27). A recent study elegantly illustrated this difference in responsiveness between individuals with different levels of aerobic capacity (24). Whereas low and moderately trained athletes were shown to improve running time-trial performance after 7 days of sodium nitrate supplementation, the highly-trained group ($\dot{V}O_{2peak}$ ~72 $mL \cdot kg^{-1} \cdot min^{-1}$) did not show any improvements when compared to the placebo condition (24). Although we cannot exclude potential benefits from extending the supplementation period beyond 6 days (in line with Vanhatalo *et al.,* (11)), the present study supports current literature by showing no changes in $\dot{V}O_2$ or time-trial performance after both acute or 6-day nitrate supplementation in highly-trained athletes.

Interestingly, despite not detecting ergogenic effects at the group level, some studies have suggested that there may be specific nitrate 'responders' within the group of highly-trained athletes (14, 16). Wilkerson *et al.* (16) identified 5 of the 8 highly-trained cyclists in their study as possible 'responders' based on increases in plasma [nitrite] (+45%), and improvements in time-trial performance (2% faster). In the present study, all subjects showed substantial increases in plasma [nitrite] averaging +287±216 nM (range 94-881 nM); or +162±152%. However, no performance improvements were observed at the group level. This seems to suggest that measuring increases in plasma [nitrite] following nitrate ingestion does not suffice when identifying potential 'responders' to the ergogenic properties of nitrate supplements. Alternatively, other studies defined 'responders' solely based on improvements in time-trial performance and/or changes in $\dot{V}O_2$ (14, 28). Although we did not observe any changes in $\dot{V}O_2$, six subjects did show a faster time to complete the time-trial with both the 1-DAY (4.5±2.4% faster) and the 6-DAY (5.4±2.9% faster) protocols. Interestingly, the differences in time trial performance between trials were less pronounced (2.3±3.5%), in subjects in which time-trial performance was better following ingestion of PLA or with only one of the nitrate interventions (1-DAY or 6-DAY). However, as we previously suggested, a more robust scientific approach is needed to quantify the responsiveness to nitrate supplementation in individual athletes (29). As such, caution should be taken when speculating on potential 'responders' and 'non-responders' to nitrate supplementation based on our data.

We conclude that both acute and 6-day nitrate supplementation result in similar increases in plasma [nitrate] and [nitrite]. However, 800 mg sodium nitrate supplementation, acutely or successively for 6 days does not lower oxygen requirements during submaximal exercise or improve time-trial performance in highly trained cyclists.

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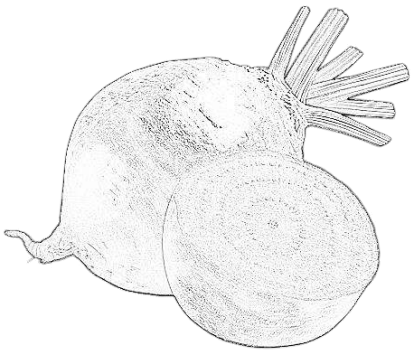
Chapter 3

Nitrate-rich vegetables increase plasma nitrate and nitrite concentrations and lower blood pressure in healthy adults



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Abstract

Background: Dietary nitrate is receiving increased attention due to its reported ergogenic and cardioprotective properties. It is currently unknown to what extent ingestion of various nitrate-rich vegetables increases post-prandial plasma nitrate and nitrite concentrations and lowers blood pressure.

Objective: We aimed to assess the impact of ingesting different nitrate-rich vegetables on subsequent plasma nitrate and nitrite concentrations and resting blood pressure in healthy normotensive individuals.

Design: Using a semi-randomized cross-over design, 11 males and 7 females (28±1 y, BMI 23±1 kg/m^2, exercise 1-10 h/wk) ingested 4 different beverages, each providing 800 mg (~12.9 mmol) nitrate: sodium nitrate ($NaNO_3$), concentrated beetroot juice, a rocket salad beverage, and a spinach beverage. Plasma nitrate and nitrite concentrations and blood pressure were determined before and up to 300 min after beverage ingestion. Data were analyzed using repeated-measures ANOVA.

Results: Plasma nitrate and nitrite concentrations increased after ingestion of all 4 beverages (P<0.001). Peak plasma nitrate concentrations were similar for all treatments ($NaNO_3$: 583±29, BR: 597±23, RS: 584±24, SP: 584±23 μmol/L). Peak plasma nitrite concentrations were different between treatments ($NaNO_3$: 580±58, BR: 557±57, RS: 643±63, SP: 980±160 nmol/L, P=0.016). Compared to baseline, systolic blood pressure declined 150 min after ingestion of BR (from 118±2 to 113±2 mmHg; P<0.001) and RS (from 122±3 to 116±2 mmHg; P=0.007), and 300 min after ingestion of SP (from 118±2 to 111±3 mmHg; P<0.001), but did not change with $NaNO_3$. Diastolic blood pressure declined 150 min after ingestion of all beverages (P<0.05), and remained lower at 300 min after ingestion of RS (P=0.045) and SP (P=0.001).

Conclusion: Ingestion of nitrate-rich beetroot juice, rocket salad and spinach effectively increase plasma nitrate and nitrite concentrations, and lower blood pressure to a greater extent than sodium nitrate. These findings show that nitrate-rich vegetables can be used as dietary nitrate supplements.

Introduction

Dietary nitrate, often consumed with beetroot juice as its carrier, has become a popular supplement due to its reported ergogenic (1-5) and cardioprotective (6-8) properties. These beneficial effects of dietary nitrate have been attributed to its capacity to increase the bioavailability of nitric oxide (NO). NO represents an important signaling molecule in the human body and plays a key role in several physiological processes by regulating blood flow, muscle contractility, glucose and calcium homeostasis, and mitochondrial respiration and biogenesis (9). Nitrate and nitrite have traditionally been viewed as inactive by-products of NO metabolism through the nitric oxide synthase (NOS) dependent pathway. However, research from the 1990s showed that a reverse pathway exists whereby nitrate and nitrite can be reduced back into NO (10, 11). There is now a general consensus that dietary nitrate ingestion can strongly increase plasma nitrate concentrations. Circulating nitrate is subsequently actively taken up by the salivary glands and concentrated in the saliva, where it can be reduced to nitrite by facultative anaerobic bacteria in the oral cavity. After swallowing, nitrite enters the circulation and can be further reduced to NO via various pathways (12-14).

Multiple studies have reported increased plasma nitrate and nitrite concentrations after ingestion of nitrate in the form of sodium nitrate (4, 5, 15-17). Similar effects have been reported using (concentrated) beetroot juice (1, 2, 18-23). Whereas there are several other nitrate-rich food sources, including green leafy and root vegetables (24), research on the pharmacokinetic and physiological effects of nitrate supplementation has mainly applied either sodium nitrate or beetroot juice as a nitrate donor. One of the consequences of the post-prandial increase in plasma nitrate and nitrite availability is a decrease in resting blood pressure, which has been reported after ingestion of both sodium nitrate (7) and beetroot juice (6, 8). As of yet there is little research on the optimal way of supplementing dietary nitrate. Literature has reported a dose-response relationship between the amount of dietary nitrate ingested and the rise in plasma nitrate and nitrite concentrations, as well as the reduction in oxygen cost of submaximal exercise (18) and the reduction in blood pressure (25). Apart from the dose of nitrate ingested, it is unknown which other factors influence the bioavailability of nitrate and nitrite, and subsequent performance and clinical outcomes (26). While various nitrate-rich sources are available, no studies have directly compared to what extent the actual source of dietary nitrate may affect the pharmacokinetic and physiological effects upon ingestion.

In the present study we assessed the acute pharmacokinetic and blood pressure lowering effects of ingesting various nitrate-rich sources. Therefore, recreationally active participants ingested 800 mg nitrate provided as sodium nitrate, concentrated beetroot

juice, rocket salad, and spinach, after which plasma nitrate and nitrite concentrations and resting blood pressure were determined for up to 300 min after ingestion.

Methods

Participants and ethical approval

Twenty-two healthy, adult, and non-hypertensive participants were recruited to take part in this study, which was conducted between September and December 2014. Exclusion criteria were as follows: current or recent smoking (< 6 mo), current or recent beetroot juice (or other nitrate) supplementation (<1 mo), resting blood pressure >140/90 mmHg, BMI <18 or >30 kg/m^2, age <18 or >45 y, and consumption of chronic medications. To exclude the possible confounding effect of either extremely high or low training status on NO metabolism, recreationally active participants (exercise 1-10 h/wk) were recruited. This study was approved by the medical ethical committee of the Maastricht University Medical Centre, Maastricht, the Netherlands, followed the principles of the Declaration of Helsinki, and was registered at clinicaltrials.gov as NCT02271633. After being informed about the purpose and potential risks of the study, all participants provided written informed consent.

Study design

Using a semirandomized crossover design, we investigated the impact of ingesting 800 mg dietary nitrate provided in 4 different sources on subsequent plasma nitrate and nitrite concentrations and resting blood pressure. Over a 5-wk period, participants were required to report to the laboratory on 5 occasions, consisting of a screening session (visit 1) and 4 experimental test days (visits 2-5). The 4 sources all contained 800 mg (~12.9 mmol) nitrate, and were provided as: sodium nitrate ($NaNO_3$), concentrated beetroot juice, a rocket salad (arugula) beverage, and a spinach beverage. In order to expose all participants to vegetable beverages from the same batch, a single test day was performed for the rocket salad treatment (all participants on 1 d) and also a single test day was performed for the spinach treatment (all participants on 1 d). As such, we could only randomize the order of the other 2 treatments, and defined this procedure as 'semi-randomized'. Thus, the $NaNO_3$ and beetroot juice treatments were randomly administered before and/or after the rocket salad and spinach beverage treatments (random number generator). The test days were interspaced by a 7-d washout period.

Experimental protocol

During a screening session, eligibility for participation in the study was assessed. Standard medical questionnaires were administered, and blood pressure was determined to rule out hypertension. After a 10 min rest period, blood pressure was measured 4 times using

an automated cuff (Omron Healthcare Inc, Field Court Lake Forest, USA), with the last 3 measurements being averaged to obtain mean blood pressure. Body mass (digital balance scale; accuracy 0.1 kg) and height (wall-mounted stadiometer; accuracy 0.1 cm) were measured with participants standing barefoot and dressed lightly.

On the 4 experimental test days, the participants arrived in the morning after an overnight fast. After a 10 min rest period, blood pressure was measured as described above. Subsequently, a catheter was inserted into an antecubital vein for repeated venous blood draws. After a baseline blood sample was obtained, participants consumed a standardized breakfast, immediately followed by the treatment beverage. Repeated blood draws were performed at 30, 60, 120, 150, 180, 240, and 300 min after the ingestion of the beverages. 300 min was chosen to capture the peak and the subsequent begin of decline in plasma nitrate and nitrite concentrations (26), without the need for subsequent additional food intake (i.e., lunch time). Resting blood pressure was also measured at 150 min and 300 min after beverage ingestion. In addition, a gastrointestinal tolerance questionnaire was administered at baseline and at 150 min after beverage ingestion. To limit any effect of circadian blood pressure fluctuation throughout the day (27), all test days were performed at the exact same time of the day for each participant (all participants starting between 0800 and 0900).

Study treatments

With previous work suggesting a minimally required dose of ~500 mg nitrate to induce acute blood pressure lowering effects (6, 7, 15, 25, 28, 29), and dose-response relations observed for the improvement in blood pressure (25) and other physiological parameters (18), we applied a treatment dose of 800 mg nitrate to optimize the chance of detecting effects on blood pressure. Though above the current Acceptable Daily Intake (ADI) level of 3.7 mg/kg/d (30), this dose falls well within the range that was used previously in acute and multi-day nitrate supplementation trials (including the above-mentioned studies), providing 496-1488 mg.

The 4 beverages were sodium nitrate ($NaNO_3$ [BASF, Ludwigshafen, Germany] dissolved in water), concentrated beetroot juice (Beet It, James White Drinks Ltd., Ipswich, UK), a fresh rocket salad (arugula) beverage, and a fresh spinach beverage. The beetroot juice, rocket salad, and spinach beverages were analyzed for nitrate content using chemiluminesence as described below for plasma analysis to ascertain that an exact dose of 800 mg nitrate was provided for each treatment. Beetroot juice was diluted 2000 times in distilled water (18 MΩ) before being analyzed for nitrate content. Both rocket salad and spinach beverages were blended into a smoothie-like beverage upon arrival from the store.

Adapted from a previously described method (31), the vegetable beverages were extracted for nitrate and nitrite analysis. 500 mg of beverage was weighed accurately in a screw cap glass tube. Then 5 mL distilled water and 5 mL methanol were added, and the tube was capped and shaken vigorously for 15 min, before being centrifuged (4 °C) for 5 min at 1,000 *g*. The supernatant was filtered through a PVDF filter (0.22 μm), and the filtrate was diluted in distilled water until the concentrations fell within the measurement range, before being analyzed for nitrate and nitrite content. Beetroot juice, rocket salad, and spinach beverages were analyzed for nitrate content in duplicate within 3% variance, and calculations were performed to determine the exact amount of beverage providing 800 mg of nitrate. This resulted in an amount of 1.1 g $NaNO_3$ (140 mL), 116 g beetroot juice (106 mL), 196 g rocket salad beverage (225 mL), and 365 g spinach beverage (400 mL). The beverages were stored at 4 °C and provided to the participants within 24 h after analysis for nitrate content. Nutritional composition of the treatments is provided **in Supplemental Table 3.1.**

Supplemental Table 3.1: Nutritional content of the 4 nitrate beverages

	Sodium nitrate	Beetroot juice	Rocket salad	Spinach
Weight, *g*	1.1	116	196	365
Volume, *mL*	140	106	225	400
Energy, *kJ*	0	490	192	394
Energy, *kcal*	0	115	45	95
Protein, *g*	0	4.1	6.9	11.7
Carbohydrate, *g*	0	24.2	0.2	3.3
Fat, *g*	0	0.2	1.0	2.2
Fiber, *g*	0	<0.5	4.3	7.3
Calcium, *mg*	0	9.6	531.2	383.3
Iron, *mg*	0	0.4	5.1	7.3
Potassium, *mg*	0	1073	894	1967
Vitamin C, *mg*	0	1.0	3.9	3.7
Sodium, *mg*	300	133	49	47
Nitrate, *mg*	800	800	800	800

Values for Beetroot juice are based on analytical report of Beet It Sports Shot (James White Drinks Ltd, Ipswich, UK). Values for Rocket salad and Spinach are adapted from the Dutch Food Composition Database 'NEVO'.

Physical activity and dietary standardization

In the 48 h leading up to the first experimental test day (visit 2), participants recorded their dietary intake and physical activity and refrained from strenuous exercise or labor. Participants replicated their diet and physical activities in the 48 h before the following 3 test days (visits 3-5). Participants avoided caffeine and alcohol for 12 and 24 h before each test day, respectively. To prevent any attenuation in the reduction of nitrate to nitrite in

the oral cavity by commensal bacteria, participants refrained from using any antibacterial mouthwash/ toothpaste and chewing gum, and avoided tongue-scraping during the intervention period (14). No restrictions were set for the intake of nitrate-rich foods (21, 23) during the intervention period. On the evening before each test day, all participants consumed a standardized dinner that was adapted to their bodyweight (53 kJ/kg, providing 57% of energy from carbohydrate, 27% from fat, and 16% from protein, composed of a mixed meal of potato, chicken and vegetables, orange juice, crackers and yoghurt). Furthermore, all participants received the same standardized breakfast (39 kJ/kg, providing 68% from carbohydrate, 18 % from fat, and 14% from protein, composed of bread, butter, cheese, jam, crackers and orange juice) on the morning of each test day before ingestion of the beverage. The ad libitum consumption of water was registered during the first test day (visit 2), and replicated during the subsequent test days (visits 3-5).

Plasma analysis

Blood samples were collected in S-Monovette® Lithium-Heparin containing tubes (Sarstedt, Nümbrecht, Germany) for plasma nitrate and nitrite analysis, and centrifuged immediately at 1,000 *g* for 5 min, at 4 °C. Aliquots of plasma were frozen and stored at –80 °C for subsequent analysis. Determination of plasma nitrate and nitrite concentrations was performed using the chemiluminescence technique, which has been described previously (32). In short, plasma nitrate and nitrite concentrations are determined by their reduction to NO. The spectral emission of electronically excited nitrogen dioxide, from the NO reaction with ozone, is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube, housed in a Sievers gas-phase chemiluminescence NO analyzer (NOA; Sievers NOA 280i; Analytix, Durham, UK). Inter- and intra-assay CVs were 4.2% and 1.1% for plasma nitrate, and 4.9% and 4.7% for plasma nitrite.

Statistical methods

For the power calculation we used a difference in plasma nitrite concentrations of 50 nmol/L as primary outcome. With a crossover design, sample size was calculated with a power of 95%, a significance level of 0.008 (to adjust for Bonferroni corrected post-hoc testing), and a drop-out rate of 10%. The final number of participants to be included after screening was calculated to be 22. Plasma nitrate and nitrite concentrations and resting blood pressure were analyzed using a 2-factor (time × treatment) repeated measures analysis of variance (ANOVA). Additionally, 1-factor repeated-measures ANOVA was used to determine differences in peak concentrations, time to peak values and incremental area under the curve (iAUC) for both plasma nitrate and nitrite concentrations, between the different treatments. For each treatment, peak concentrations were defined as the

highest measured values at any time point for each individual. Time to peak was defined as the time point where the individual reached the highest plasma nitrate and nitrite concentrations. iAUC was calculated by multiplying each time period (i.e., 30 or 60 min) by the average increase above baseline for that period, summing all time periods. This was done for each individual and each treatment separately. Although we did not specifically aim to study any sex effects on the response to dietary nitrate ingestion, the individual plasma nitrate and nitrite graphs appeared substantially different between men and women upon visual inspection. Therefore, as a secondary analysis, we included sex as a between-subjects factor in the ANOVA analyses described above. As another secondary analysis, the change in blood pressure from baseline was compared between groups using 1-factor repeated measures ANOVA. For all analyses, statistical significance was set at $P<0.05$, and any interaction or main effect was subsequently analyzed using a Bonferroni's corrected post hoc test. All data were analyzed using SPSS 22.0 (SPSS Inc., USA), and are presented as means ± SEMs.

Results

Four of the 22 participants dropped out during the study due to failure to comply with the protocol (incomplete ingestion of 1 of the beverages, within the given timeframe of 15 min). General characteristics of the remaining 18 participants are reported in **Table 3.1**.

Table 3.1: Participants' characteristics of healthy adults who ingested 4 different nitrate sources[1]

	All	Men	Women
n	18	11	7
Age, *y*	28±1	29±2	26±2
Height, *cm*	181±2	185±2	174±3
Body mass, *kg*	76±3	82±3	66±4
BMI, kg/m^2	23±1	24±1	22±1

[1]Values are means±SEM. BMI: body mass index.

Plasma nitrate

Plasma nitrate concentrations at different time points are presented in **Figure 3.1A**. Baseline plasma nitrate concentrations were similar for all 4 treatments ($NaNO_3$: 66±6 μmol/L, beetroot juice: 61±5 μmol/L, rocket salad beverage: 63±6 μmol/L, and spinach beverage: 69±6 μmol/L, $P=0.56$). A significant time x treatment interaction ($P<0.001$) was observed; although plasma nitrate increased to similar peak concentrations after ingestion of all 4 beverages ($NaNO_3$: 583±29 μmol/L, beetroot juice: 597±23 μmol/L, rocket salad beverage: 584±24 μmol/L, and spinach beverage: 584±23 μmol/L, $P=0.65$), differences in nitrate concentrations were observed at specific time points (t=30, 60, 120, 240, and 300 min; **Figure 3.1A**). Furthermore, there was a significant treatment effect for

time to peak (*P*<0.001; **Figure 3.1B**), and post hoc analysis showed that after ingestion of rocket salad and spinach beverages, time to peak was later compared to both $NaNO_3$ and beetroot juice (all *P*<0.001). As a result, the iAUC was significantly different between the treatments (*P*<0.001; **Figure 3.1C**). Post hoc analysis showed that the iAUC for plasma nitrate concentrations after ingestion of $NaNO_3$ was smaller than beetroot juice (*P*=0.038), greater than rocket salad beverage (*P*=0.007), but not different from spinach beverage (*P*=0.082). Moreover, iAUC for plasma nitrate after ingestion of beetroot juice was higher than both rocket salad and spinach beverages (both *P*<0.001).

Plasma nitrite

Plasma nitrite concentrations at different time points are presented in **Figure 3.2A**. Baseline plasma nitrite concentrations were similar for all 4 treatments ($NaNO_3$: 131±24 nmol/L, beetroot juice: 135±20 nmol/L, rocket salad beverage: 115±14 nmol/L, and spinach beverage: 155±25 nmol/L, *P*=0.37). A significant time x treatment interaction (*P*=0.001) showed that although plasma nitrite increased after ingestion of all 4 beverages (all *P*<0.001), differences in nitrite concentrations were observed at specific time points (t=30, 60, 120, 150, and 180 min; Figure 3.2A). There were no significant differences in time to peak between the treatments (*P*=0.077; **Figure 3.2B**). However, there was a significant treatment effect for peak nitrite concentrations ($NaNO_3$: 580±58 nmol/L, beetroot juice: 557±57 nmol/L, rocket salad beverage: 643±63 nmol/L, and spinach beverage: 980±160 nmol/L, *P*=0.016). Although peak nitrite concentration was numerically greater for the spinach beverage, differences were not significant in the post hoc test (*P*=0.084, *P*=0.122, and *P*=0.197, for spinach beverage compared with beetroot juice, $NaNO_3$, and rocket salad beverage, respectively). The iAUC for plasma nitrite concentrations was significantly different between treatments (*P*=0.003; **Figure 3.2C**), and was higher after ingestion of spinach beverage compared with $NaNO_3$ (*P*=0.025), beetroot juice (*P*=0.035) and rocket salad beverage (*P*=0.046), with no differences between $NaNO_3$, beetroot juice, and rocket salad beverage.

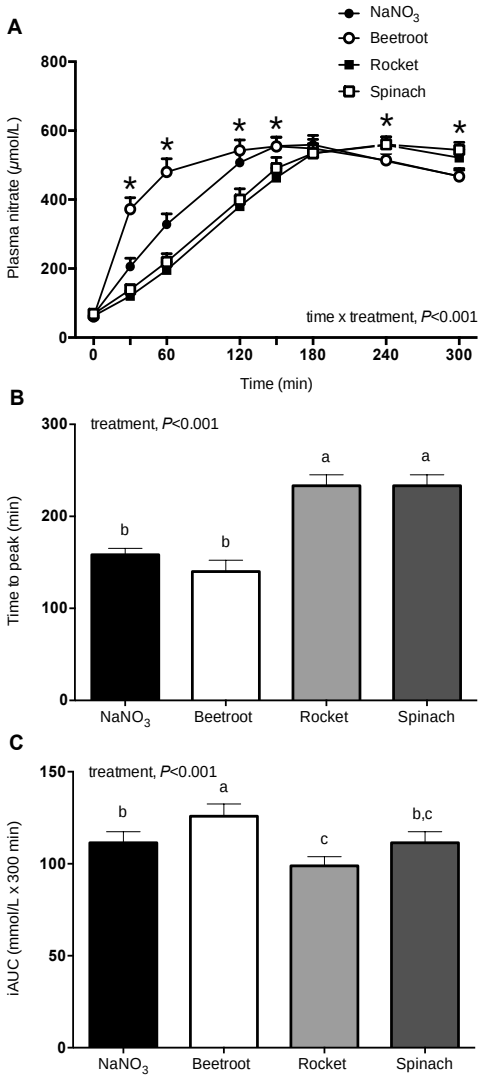


Figure 3.1. Plasma nitrate concentrations (A), time to peak plasma nitrate concentrations (B), and incremental area under curve (iAUC) for plasma nitrate concentrations (C) in 18 healthy adults ingesting 4 different nitrate sources. Values are means±SEM. A: *Significant difference between treatments, P<0.05. B-C: a>b>c: labeled means without a common letter differ, P<0.05.

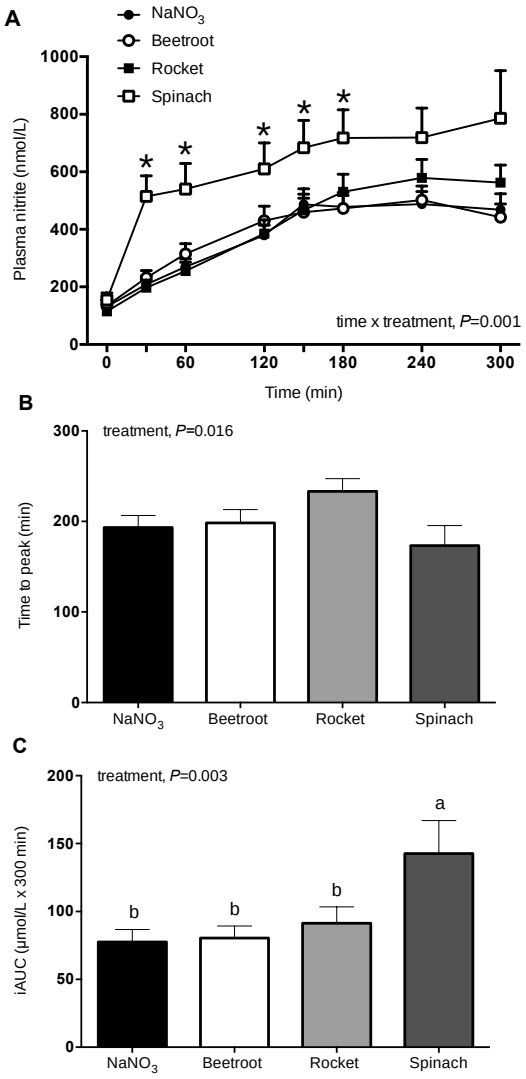


Figure 3.2. Plasma nitrite concentrations (A), time to peak plasma nitrite concentrations (B), and incremental area under curve (iAUC) for plasma nitrite concentrations (C) in 18 healthy adults ingesting 4 different nitrate sources. Values are means±SEM. A: *Significant difference between treatments, P<0.05. B-C: a>b: labeled means without a common letter differ, P<0.05.

Blood pressure

A significant time x treatment interaction was observed for both systolic (*P*=0.017; **Figure 3.3A**) and diastolic (*P*=0.010; **Figure 3.3B**) blood pressure. Separate analyses showed a decrease in systolic blood pressure from baseline to 150 min following ingestion of both beetroot juice (*P*<0.001) and rocket salad (*P*=0.007; **Figure 3.3C**). After ingestion of spinach beverage, systolic blood pressure was significantly lower at 300 min compared to

baseline values (*P*<0.001; Figure 3.3C). In contrast, no changes in systolic blood pressure were observed after ingestion of $NaNO_3$ (*P*=0.11, Figure 3.3C). The change in systolic blood pressure from baseline to 150 min (Figure 3C) was significantly different between treatments (*P*=0.022), with post-hoc analysis showing a significant difference for $NaNO_3$ compared with beetroot juice (*P*=0.022) and $NaNO_3$ compared with rocket salad beverage (*P*=0.001). No differences were observed in the change in systolic blood pressure from baseline to 300 min between treatments (*P*=0.21), despite the strong reduction for the spinach beverage treatment only (Figure 3.3C).

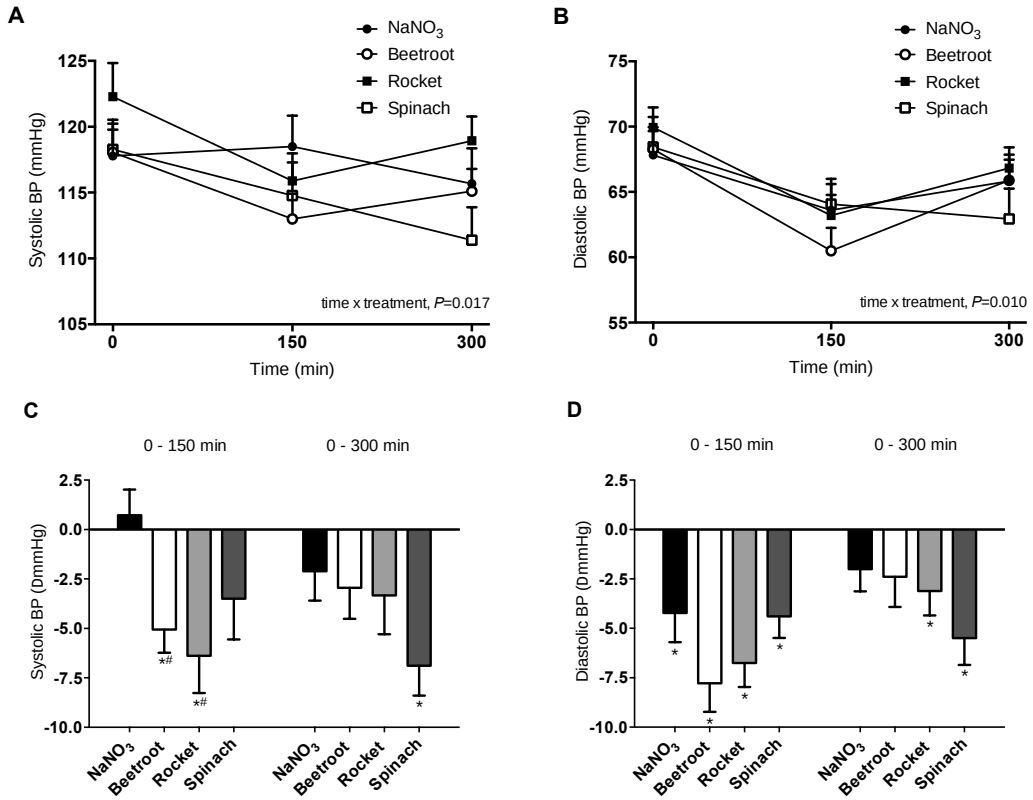


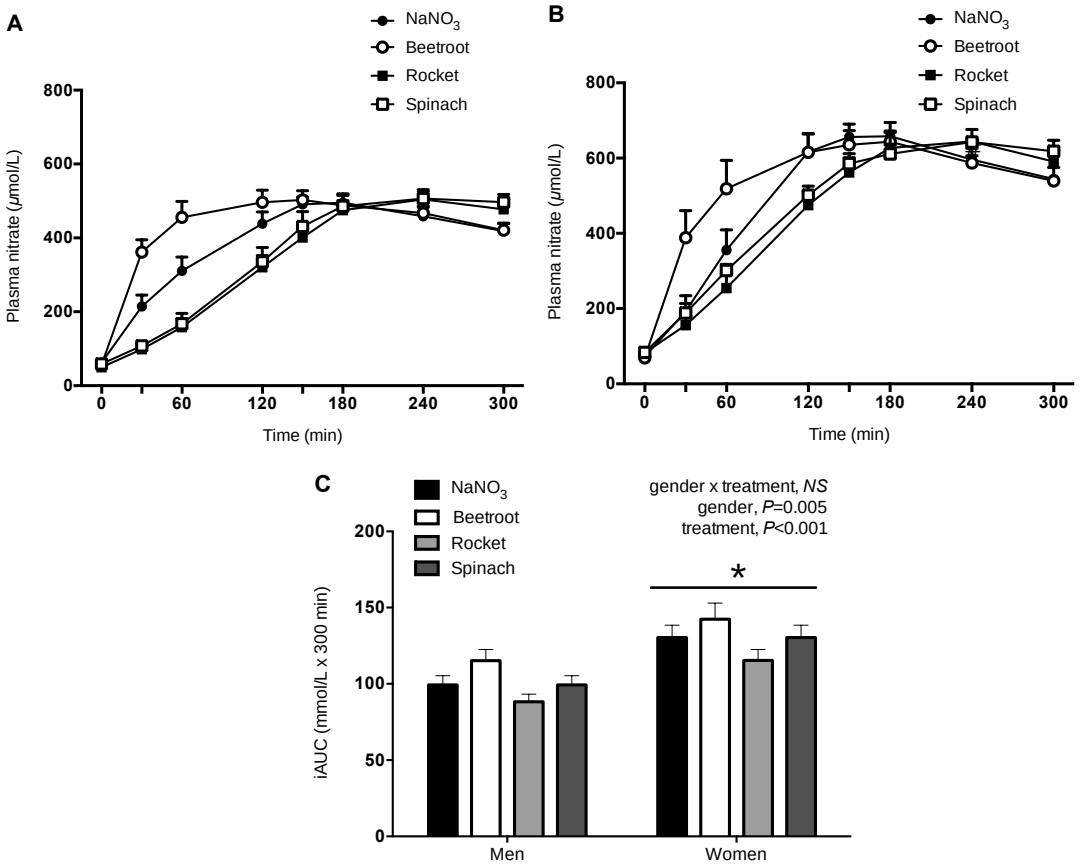
Figure 3.3. Blood pressure reported as absolute systolic (A), absolute diastolic (B), change in systolic from baseline (C) and change in diastolic from baseline (D) in 18 healthy adults ingesting 4 different nitrate sources. Values are means ±SEM. For reasons of clarity, treatment differences are only displayed in panels C-D. *Significant change from baseline within treatment, *P*<0.05. #Change from baseline significantly different compared to $NaNO_3$, *P*<0.05.

For diastolic blood pressure, separate analyses showed a decrease from baseline to 150 min following ingestion of all beverages ($NaNO_3$; *P*=0.022, beetroot juice and rocket salad beverage; *P*<0.001, and spinach beverage; *P*=0.002; **Figure 3.3D**). However, at 300 min after ingestion, diastolic blood pressure only remained significantly lower compared to baseline values in the rocket salad beverage (*P*=0.045) and spinach beverage (*P*=0.001; Figure 3.3D) treatments. Despite these observations, no differences were observed in the

actual change in diastolic blood pressure from baseline to 150 and 300 min between treatments (*P*=0.12 and *P*=0.23, respectively).

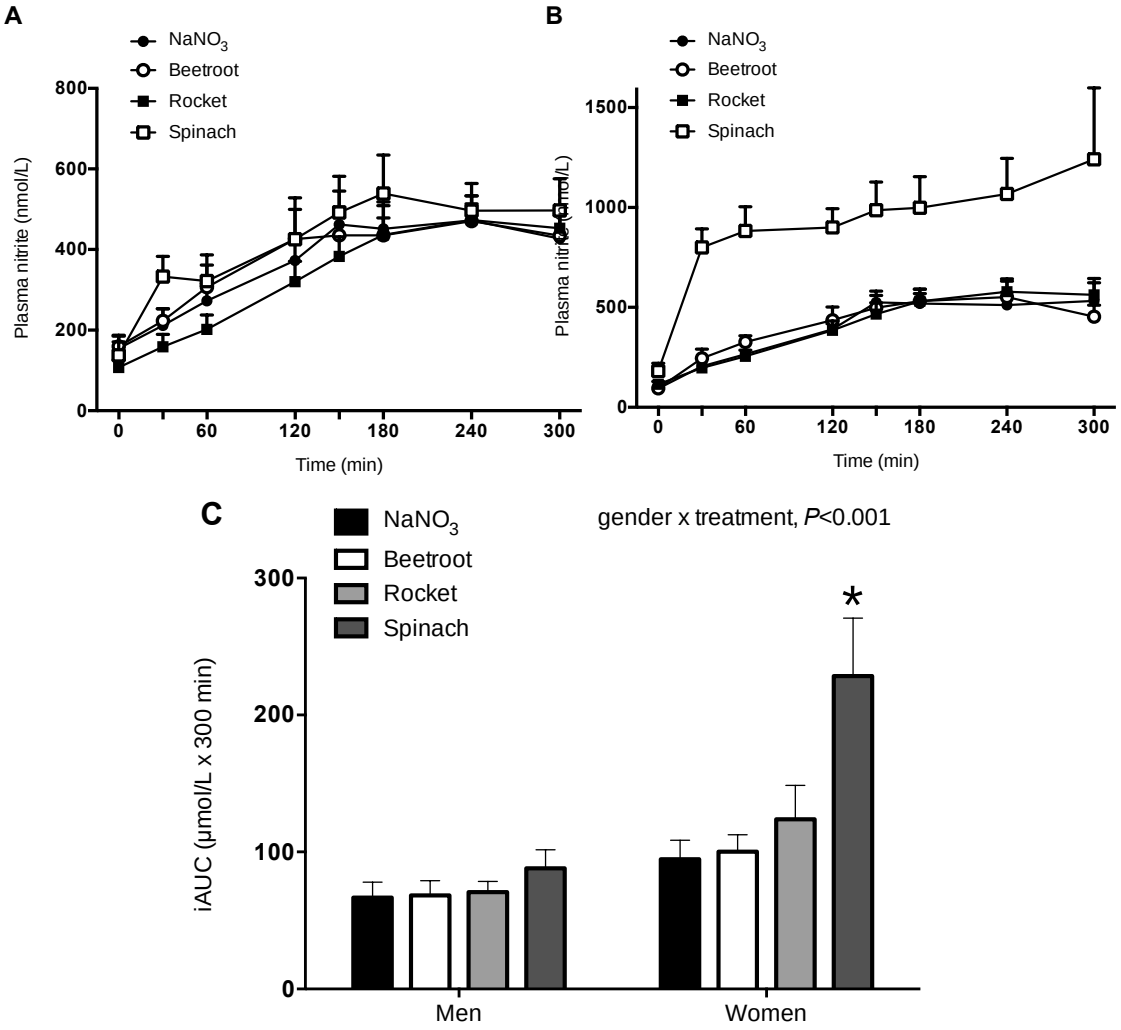
Sex

Plasma nitrate and nitrite concentrations and iAUC for men and women separately are presented in **Supplemental Figure 3.1** and **Supplemental Figure 3.2**.



Supplemental Figure 3.1. Plasma nitrate concentrations before and up to 300 min after ingestion of 4 different nitrate sources in men (n=11, A) and women (n=7, B), and iAUC for plasma nitrate in men vs women (C). Values are means±SEM. *Significantly different from men. For post-hoc analysis of the treatment effect, see Figure 4.1C.

Sub-analysis including sex as a between-subjects factor showed that the baseline (*P*=0.020), the peak value (*P*<0.001), and the iAUC (*P*=0.005) for plasma nitrate concentrations were higher in the women compared to men for all 4 treatments. For plasma nitrite concentrations, no sex differences were observed at baseline.



Supplemental Figure 3.2. Plasma nitrite concentrations before and up to 300 min after ingestion of 4 different nitrate sources in men (n=11, A) and women (n=7, B), and iAUC for plasma nitrite in men vs women (C). Values are means±SEM. *Significantly different from men. For post-hoc analysis of the treatment effect, see Figure 4.2C.

Although peak values and iAUC for plasma nitrite concentrations appeared higher in the women than the men, this difference reached statistical significance for only the spinach beverage treatment (P=0.010 and P=0.016, respectively). Despite these sex differences in the plasma nitrate and nitrite responses, no differences in blood pressure responses to nitrate ingestion were observed between men and women.

Side effects

No serious adverse events were reported. Ingestion of the 4 beverages was well tolerated by most of the participants. Four participants (1 man and 3 women) did not manage to ingest the total volume of the rocket salad or the spinach beverage within the given timeframe of 15 min, and were therefore excluded from the study. Gastrointestinal complaints were reported by 2 participants after the rocket salad beverage, and by 1

participant after the spinach beverage. No gastrointestinal complaints were reported for the $NaNO_3$ and beetroot juice beverages, respectively. One participant reported headache at baseline and throughout the test day after ingestion of the spinach beverage.

Discussion

The present study demonstrated that acute ingestion of 800 mg nitrate from beetroot juice, rocket salad and spinach elevated plasma nitrate concentrations to the same extent (~9 fold) as the ingestion of an identical dose from sodium nitrate. Although ingestion of all 4 nitrate sources led to substantial increases in plasma nitrite concentrations, the increase was greater (~6 fold vs ~4 fold) after ingestion of spinach compared to the ingestion of the other nitrate sources. In contrast to the ingestion of sodium nitrate, ingestion of beetroot juice, rocket salad and spinach resulted in a significant lowering of both systolic and diastolic blood pressure in this group of healthy, normotensive individuals.

Over the past decade, beetroot juice has become an increasingly popular dietary supplement due to its high nitrate content, and the associated ergogenic and cardioprotective properties. Research in this area has mainly utilized the ingestion of beetroot juice and sodium nitrate as dietary intervention. Both sources have been shown to substantially increase plasma nitrate and nitrite concentrations, improve exercise performance (1-5), and lower blood pressure (6-8) in healthy individuals. However, it remains unclear whether differences exist in the pharmacokinetic and physiological responses to the ingestion of different dietary nitrate sources. We assessed the acute effects of ingesting various nitrate-rich sources on plasma nitrate and nitrite concentrations and resting blood pressure. In our study, ingestion of exactly 800 mg of nitrate from all 4 sources led to similar increases (~9 fold) in plasma nitrate concentrations (Figure 3.1A). Our data lend further support to the suggestion by Van Velzen *et al* (33) that dietary nitrate from different vegetable sources can be effectively absorbed, reaching ~100% bioavailability in the circulation. The time to peak plasma nitrate concentrations after sodium nitrate and beetroot juice ingestion (Figure 3.1B) was similar to that shown in earlier studies using these sources (18, 32). The longer time needed to reach peak plasma nitrate concentrations after ingestion of rocket salad and spinach may be related to their higher fiber content and the larger volume ingested (Supplemental Table 1), likely resulting in a slower gastric emptying (34). Hence, the optimal timing of supplementation may slightly differ between various nitrate sources. More importantly though, ingestion of dietary nitrate from all 4 sources seemed to be equally effective in increasing plasma nitrate concentrations.

In line with the data on plasma nitrate concentrations, substantial increases in plasma nitrite concentrations were observed after ingestion of all 4 beverages (Figure 2A). Both the increases in plasma nitrite concentrations (~4 fold) and time to reach peak values (~180-240 min) are in agreement with previous work supplementing ~500-1000 mg nitrate in the form of sodium nitrate (15) or beetroot juice (2, 20-22). In contrast with the plasma nitrate concentrations, time to reach peak plasma nitrite concentrations did not differ between treatments (Figure 2B). This could imply that the speed of bioconversion of nitrate to nitrite is not merely driven by the rate at which nitrate appears in the circulation. Furthermore, the total release of nitrite into the blood may be affected by the composition of the specific nitrate source. In this context, we observed a remarkable initial increase in plasma nitrite concentrations after ingestion of spinach (Figure 3.2A), which was much stronger compared to the other beverages. This substantial increase in plasma nitrite (~4 fold within 30 min) is unlikely to be completely explained by the endogenous conversion of dietary nitrate (32). Alternatively, the initial increase in plasma nitrite after spinach ingestion may be attributed to the higher nitrite content found in spinach compared to other vegetables (35). In agreement, analysis of the 4 provided beverages for nitrite content revealed substantially greater nitrite content in the spinach beverage (~100 μg) compared to the rocket salad beverage (~20 μg), beetroot juice (<1 μg) and sodium nitrate (<1 μg). Though this may partly explain the greater initial increase in plasma nitrite concentrations after ingestion of the spinach, plasma nitrite concentrations continued to increase throughout the test day. In fact, the increase in plasma nitrite concentrations from 30 min after ingestion to peak values was similar for all 4 beverages (Figure 3.2A). As such, our findings are the first to show that the effective uptake of dietary nitrate and the total *bioconversion* of the ingested nitrate into nitrite do not seem to depend on the source of dietary nitrate.

Plasma nitrite represents a precursor for the NOS-independent formation of NO, which can then act as a vasodilatory agent thereby lowering blood pressure (6). Previous work in healthy populations has shown reductions in blood pressure after acute beetroot juice (6, 8, 20, 22, 29), several-day beetroot juice (1, 2, 23) or acute spinach (36, 37) ingestion. The blood pressure lowering effects of sodium nitrate have been studied less extensively. We are the first to compare the blood pressure lowering effects after ingestion of various nitrate-rich vegetable sources and sodium nitrate, using identical nitrate doses. We observed a more pronounced decrease in blood pressure after ingestion of the vegetable beverages (systolic ~5-7 mmHg, diastolic ~4-8 mmHg; Figure 3C and 3D) compared to sodium nitrate (only diastolic, ~2-4 mmHg). In line with our findings, previous work reported that despite substantial increases in plasma nitrate and nitrite concentrations, sodium nitrate only decreased diastolic blood pressure while systolic blood pressure

remained unaffected (7, 15). It has been speculated that other compounds in vegetables (such as vitamin C, potassium and polyphenols) may contribute and/or act synergistically with nitrate, enhancing its blood pressure lowering effects. For example, it has been shown that vitamin C increases the reduction of nitrite to NO (38, 39), which may potentiate the blood pressure lowering effects at a given nitrate/nitrite concentration. Additionally, 2 studies using potassium nitrate observed positive effects on both systolic and diastolic blood pressure (25, 28), suggesting that the combination of potassium and nitrate may be more effective than sodium and nitrate. Obviously, all vegetable beverages in the present study contained substantial amounts of macro- and micronutrients that were not present in the sodium nitrate beverage (Supplemental Table 3.1). As such, the principle of a synergistic effect between nitrate and other nutritional compounds may explain why the vegetable beverages are more effective than sodium nitrate in lowering blood pressure, despite similar increases in plasma nitrate and nitrite concentrations. Yet, it remains to be established what specific mechanisms may underlie a potentially more effective conversion of nitrite into bio-active NO, thereby explaining the more pronounced blood pressure lowering effects following ingestion of different nitrate-rich vegetables.

A discordance between changes in plasma nitrite concentrations and subsequent physiological effects was recently also observed by Larsen *et al* (17). Despite a substantial increase in plasma nitrite concentrations, intravenous nitrite infusion did not lead to a decreased resting metabolic rate as observed after ingestion of dietary nitrate (17). In accordance, the initial high plasma nitrite concentrations after spinach ingestion did not translate into any additional reduction in blood pressure in the present study (Figure 3.3C). Overall, it appears that plasma nitrite *per se* is not the key driver, nor the key indicator of the physiological effects induced through the nitrate-nitrite-NO pathway. Though nitrite represents an important intermediate, Larsen *et al* (17) suggest that nitrate-derived bioactive nitrogen oxides other than nitrite could be the final mediators of the observed effects. Ultimately, it is the actual bio-availability of NO that is likely driving any physiological effects (32). In this respect, cyclic guanosine mono-phosphate (cGMP) has been suggested to be the most sensitive indicator of NO bio-activity (25). As such, cGMP should be included in future studies investigating the effects of dietary nitrate, including potential differences between various dietary nitrate sources.

The major strength of the present study lies in the within-subject comparison of the 4 treatments, providing exactly 800 mg of nitrate with each treatment, under fully standardized conditions. By testing each participant at the same time of day for all 4 treatments, we bypassed the effects of circadian fluctuation of blood pressure, ensuring a valid comparison between treatments. However, since blood pressure is known to rise

throughout the morning (27), we cannot rule out the possibility that we may have overlooked (in the case of sodium nitrate) or simply underestimated the blood pressure lowering effect of each treatment. We chose not to burden the participants with a control trial since our primary aim was to study the pharmacokinetics of plasma nitrate and nitrite. It is clearly established that plasma nitrate and nitrite do not change upon placebo ingestion (20, 22, 40, 41). As a further limitation, we did not power to investigate any potential sex differences. However, sub-analysis of the data indicated higher plasma nitrate and nitrite concentrations in women compared with men (Supplemental Figures 3.1 and 3.2). Of note, these sex differences did not translate to any differences in the blood pressure lowering effects after nitrate ingestion. The sex differences in plasma nitrate and nitrite concentrations could partly be explained by differences in body mass and/or distribution volume of plasma. Previous studies have also reported higher plasma nitrate and/or nitrite responses in women versus men, while a subsequent reduction in platelet reactivity and increase in platelet cGMP (42), as well as a reduction in blood pressure (25) was more pronounced in men versus women. As a potential explanation, it has been suggested that there may be sex differences in the bacterial colonization of the tongue, likely affecting nitrate reductase activity (25). Again, it appears that not nitrate/nitrite *per se*, but rather the effective bioconversion into NO is the final mediator of any physiological effects. As such, sex differences in the response to dietary nitrate ingestion, including cGMP, may represent a key target for future research.

The present work clearly shows that various natural food sources, here provided as vegetable beverages, can be used effectively as dietary nitrate donors, with no differences in post-prandial plasma nitrate and nitrite concentrations. Furthermore, all vegetable beverages induced a substantial reduction in blood pressure, despite the recruitment of healthy, normotensive individuals. Our findings are relevant for the development of nutritional strategies to increase dietary nitrate intake either to enhance sports performance or facilitate clinical and/or health benefits (43). Obviously, the minimal nitrate dose needed to achieve beneficial effects should be investigated. We provided a dose exceeding the recommended ADI of 3.7 mg/kg/d (30), but well within the range used in studies showing blood pressure lowering effects (6, 7, 15, 25, 28, 29). There is an ongoing debate on potential health risks of long-term exposure to high nitrite and nitrate, especially with regard to the formation of low-molecular-weight N-nitrosamines, and associated cancer risk. Given the ‘acute’ setting in the present study (i.e., determining plasma and blood pressure responses up to 300 min after a single nitrate dose), we chose not to burden our participants with multiple 24-hour urine collections to measure nitroso-compound formation. Furthermore, previous work suggests that there is no relevance of measuring nitroso formation in plasma after acute nitrate supplementation (32). Though

the risk of long-term exposure to nitrite and nitrate and developing cancers is weak at best, and the cardiovascular benefits have been suggested to outweigh the risks (43), any potential risk still needs to be carefully considered. Therefore, we propose that future work investigating (long-term) exposure to high nitrate intakes should include measurements of nitroso-compound formation. In the present study, we did not observe any acute serious adverse effects. A few participants reported minor gastrointestinal complaints after ingestion of the rocket salad or spinach beverage, likely related to the total volume to be ingested rather than the nitrate dose. For practical use of various nitrate sources, future studies should focus on optimizing the volume, consistency and palatability of various nitrate sources.

In conclusion, ingestion of 800 mg nitrate provided as sodium nitrate, beetroot juice, rocket salad, and spinach substantially increases plasma nitrate and nitrite concentrations. Ingestion of nitrate from beetroot juice, rocket salad and spinach lowers blood pressure to a greater extent than ingestion of the same amount of nitrate provided as sodium nitrate. These findings imply that nitrate-rich vegetables can be used as dietary nitrate supplements.

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Chapter 4

Beetroot juice supplementation improves high-intensity intermittent-type exercise performance in trained soccer players

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Abstract

Background: It has been shown that nitrate supplementation can enhance endurance exercise performance. Recent work suggests that nitrate ingestion can also increase intermittent type exercise performance in recreational athletes. We hypothesized that six days of nitrate supplementation also improves high-intensity intermittent type exercise performance in trained soccer players.

Methods: Thirty two male soccer players (age: 23±1 y, height: 181±1 cm, weight: 77±1 kg, playing experience: 15.2±0.5 y, playing in the first team of a 2nd or 3rd Dutch amateur league club) participated in this randomized, double-blind cross-over study. All subjects participated on two test days in which high-intensity intermittent running performance was assessed using the Yo-Yo IR1 test. Subjects ingested nitrate-rich (140 mL; ~800mg nitrate/day; BR) or a nitrate-depleted beetroot juice (PLA) for six subsequent days, with at least eight days of wash-out between trials. The distance covered during the Yo-Yo IR1 was the primary outcome measure, while heart rate (HR) was measured continuously throughout the test, and a single blood and saliva sample were collected just prior to the test.

Results: Six days of BR ingestion increased plasma and salivary nitrate and nitrite concentrations in comparison to PLA (P<0.001), and enhanced Yo-Yo IR1 test performance by 3.4±1.3% (from 1,574±47 to 1,623±48 m; P=0.027). Mean HR was lower in the BR (172±2) vs PLA trial (175±2; P=0.014).

Conclusion: Six days of BR ingestions effectively improves high-intensity exercise performance in trained soccer players.

Introduction

While nitrate and nitrite were previously considered inert byproducts of the nitric oxide (NO) metabolism, recent insights suggest that (dietary) nitrate can also serve as a precursor for NO through the nitrate → nitrite →NO-pathway (1). Different studies have shown that both plasma nitrate and nitrite concentrations increase following dietary nitrate supplementation in a dose-dependent manner (2, 3). These elevations in plasma concentrations have in turn been associated with improvements in exercise performance, suggesting ergogenic benefits from activation of the nitrate to NO pathway (4-6).

Multiple studies from different laboratories have shown that dietary nitrate ingestion can decrease the oxygen cost of submaximal exercise and increase high-intensity exercise tolerance in recreational athletes (4, 7, 8). Furthermore, we have previously shown that nitrate-rich beetroot juice ingestion can not only increase oxygen efficiency during submaximal cycling exercise, but that it can also improve time trial performance in moderately trained cyclists and triathletes (5). As such, this work, in line with others (6, 9), has established a functional benefit of dietary nitrate supplementation on exercise performance.

Most of the earlier work on the ergogenic effects of nitrate supplementation was focused on endurance type sports, while little attention has been given to high-intensity and/or intermittent type exercise performance. However, recent findings suggest that nitrate might largely convey its effects on exercise performance through type II muscle fibers (10, 11). Ferguson *et al.* (10) used a rat model to assess the effects of dietary nitrate supplementation on blood flow *in vivo* during submaximal exercise. The increases in blood flow and vascular conductance in the exercising limbs were primarily observed in fast twitch muscle fibers. In line with these observations, Hernandez *et al.* (11) reported that dietary nitrate supplementation improves intracellular calcium handling in fast-twitch muscles of mice, which resulted in increased force production. Based on these findings in rodents, it could be suggested that the ergogenic effects of nitrate might be most profound for activities that recruit type II muscle fibers (10, 11), i.e. (very) high-intensity exercise bouts of short duration.

Soccer is one of the world's most widely performed team sports and is characterized by players performing multiple bouts of high-intensity running and sprinting throughout the 90 minutes of a match, during which there is heavy reliance on the contribution of type II muscle fibers [(12). These periods of high-intensity activity are alternated with periods of relative recovery, resulting in an intermittent type intensity profile (12-14). The Yo-Yo

Intermittent recovery test level 1 (Yo-Yo IR1) is an often used measurement tool to simulate these soccer specific activities in a controlled setting, thereby allowing the reliable and feasible assessment of physical performance in soccer players (15). Indeed, the Yo-Yo IR1 test has been shown to cover aspects of both aerobic as well as anaerobic performance in soccer players, with a strong link towards the ability to perform high-intensity intermittent type exercise throughout a match (12, 15). Using the Yo-Yo IR1, two previous studies described improved high-intensity intermittent type exercise performance following nitrate-rich beetroot juice ingestion in recreationally active team sport players (16, 17). These observations were the first indication of ergogenic benefits that team sport players (such as soccer players) could have from nitrate ingestion. The earlier of the two studies observed these effects after ingestion of a high nitrate dose (1780 mg, 28.7 mmol) in the 30 h prior to the high-intensity intermittent running test (16). Although effective, the dosing strategy that was applied in that study strongly deviates from that of current multiday supplementation protocols that have proven effective in endurance athletes (4, 5, 7). More in line with current nitrate supplementation regimens, Thompson *et al*. recently concluded that a five day nitrate supplementation protocol with a lower daily dose of nitrate was also effective in improving high-intensity intermittent running performance in recreational athletes (17). Extending on this finding in recreational athletes, we hypothesized that a homogenous group of trained soccer players performing intermittent type exercise would also benefit from nitrate ingestion. Therefore, we assessed the effects of a six day nitrate-rich beetroot juice supplementation protocol on high-intensity intermittent running performance in a group of trained soccer players.

Methods

Subjects

A total of forty, first team, male soccer players competing in the 2nd and 3rd Dutch amateur league were recruited to participate in the study. After being informed about the purpose and potential risks of the study, all subjects provided written informed consent. The experimental protocol and procedures were approved by the medical ethical committee of the Maastricht University Medical Centre, the Netherlands (METC 153006; ClinicalTrials.gov: NCT02436629). Eight subjects failed to complete the study because of injury (*n*=3), failure to comply with the protocol (dietary/activity standardization procedures; *n*=4), or due to personal time constraints (*n*=1). Data of the remaining 32 subjects (age: 23±1 y, height: 181±1 cm, weight: 77±1 kg, BMI: 23.4±0.4 kg/m^2, playing experience: 15.2±0.5 y) was used in the analysis.

Study design

This double blind, randomized, placebo-controlled, cross-over study was designed to investigate whether six days of nitrate-rich beetroot juice (BR) supplementation improves intermittent type exercise performance in trained soccer players. Subjects were required to report to the research facility on four occasions, spread over a three week period. Following a screening session (visit one), subjects visited the research facility ~1 week prior to the first experimental trial to get familiarized with the Yo-Yo intermittent Recovery test level 1 (Yo-Yo IR1) and to receive their supplemental beverages (visit two). No blood or saliva samples were collected during familiarization. The experimental trial days (visits three and four) that followed were each on day six of the nitrate-rich or nitrate-depleted beetroot juice supplementation period, with the last supplemental bolus being ingested 3 h prior to performing the Yo-Yo IR1. Wash-out between the two supplementation periods was at least eight days.

Supplementation protocol and standardization of physical activity and diet

During the two 6-day supplementation periods, subjects ingested 2x70 mL/day of beetroot juice. The choice for beetroot juice was largely based on previous observations by us (18), and by others (19), that suggest more pronounced benefits from nitrate ingestion through plant based sources than following sodium nitrate ingestion. The daily 140 mL bolus of nitrate-rich beetroot juice (BR) provided ~800 mg of nitrate (~12.9 mmol), while the beetroot juice placebo (PLA) was similar in taste and appearance but instead was depleted of nitrate (both supplied by Beet It, James White Drinks Ltd., Ipswich, UK). Subjects were instructed to ingest the 2x70 mL shots around the same time each day (~5 pm), which was based on the time the final bolus was ingested on day six of supplementation; i.e., 3 h prior to the exercise test. In addition, subjects recorded their activities and dietary intake in the 36 h prior to the first experimental trial, which were then replicated in the 36 h prior to the second trial. Subjects refrained from strenuous physical exercise or labor in the 48 h leading up to the trial days, and did not consume caffeine or alcohol in the 12 h and 24 h prior to each trial, respectively. To prevent any attenuation in the reduction of nitrate to nitrite by commensal bacteria in the oral cavity, subjects refrained from using antibacterial mouthwash/toothpaste and chewing gum during the six day supplementation periods (20). No restriction was set for the consumption of nitrate-rich foods. This was done to allow for the determination of the additional effect of dietary nitrate on performance, on top of the normal diet. As has also been done previously (21), on test days, all subjects were provided with a standardized dinner that was consumed ~3.5 h prior to the exercise test. After consumption of this meal and the final supplemental bolus, subjects were only allowed to consume an *ad libitum*

amount of water in the hours that followed. The amount of water consumed before and during the first trial was replicated during the second trial.

Experimental Protocol

On the last day of each supplementation period, subjects reported to the research facility ~2 h after ingesting the last 140 mL bolus of beetroot juice. The trials started with collection of a single antecubital venous blood sample and collection of a saliva sample for determination of pre-exercise nitrate and nitrite concentrations (2.5 h after ingesting the last supplemental bolus). Subjects then filled out a gastrointestinal (GI) tolerance questionnaire to assess GI complaints as a result of supplement ingestion. A heart rate monitor (Zephyr Technology Corporation, Annapolis, MD, US) was then fitted before subjects performed a standardized 10-minute warm-up, after which the Yo-Yo IR1 was performed. Heart rate was monitored continuously (1 Hz) to calculate mean heart rate throughout the test, as well as peak heart rate reached near the end of the Yo-Yo IR1 (30-s peak heart rate).

The warm-up and the Yo-Yo IR1 were performed indoors in a sports hall, on a 2 by 20 m running lane that was marked by cones, as described previously by Krustrup *et al*. (15). The test consisted of repeated 2x20 m sprints between a starting, turning, and finishing line at a progressively increasing speed controlled by audio bleeps from an audio system. Between each 2x20 min run, subjects had a 10-s active recovery period in an area of 5x2 m that was marked by cones behind the start/finishing line. When a subject failed to cross the finish line before the final bleep, a warning was given. When a subject failed to cross the finish line before the bleep for a second time, the final distance covered was registered and represented the end result (15). Immediately after completing the Yo-Yo IR1, subjects rated their perceived exertion on a Borg 6–20 scale (22).

Plasma and saliva analysis

Blood samples were collected in S-Monovette® Lithium-Heparin containing tubes (Sarstedt, Nümbrecht, Germany) and immediately centrifuged at 1,000 g for 5 min, at 4 °C. Aliquots of plasma were frozen in dry-ice after centrifugation, and were stored at -80 °C for subsequent analysis of plasma nitrate and nitrite concentrations. Saliva samples were collected in 2 mL Eppendorf cups and stored at -80°C until nitrate and nitrite concentrations were determined in both saliva and plasma using chemiluminescence, as described previously (18).

Statistical analysis

A sample size of 40 subjects, including a 20% dropout, was calculated with a power of 80% and an alpha of 0.05 (two-sided) to detect a 4.2% difference in the distance covered during the Yo-Yo IR1 between BR and PLA. Performance data from the Yo-Yo IR1, heart rate, and plasma and saliva data were analyzed with a paired samples t-test (BR vs PLA). Effect size of Yo-Yo IR1 performance was determined using Cohen's d_z statistical calculation for paired samples. Heart rate data of 7 subjects was incomplete (due to technical problems and/or shifting of the chest bands) and was therefore not included in the analysis. Pearson correlation coefficients were calculated to assess whether differences in plasma or saliva nitrate and nitrite concentrations between trials were associated with the difference in Yo-Yo IR1 performance or heart rate variables between BR and PLA. Statistical significance was set at $P<0.05$, and all data were analyzed using SPSS 21.0 (version 21.0, IBM Corp., Armonk, NY, USA), and are presented as means ± SEM.

Results

Plasma and saliva nitrate and nitrite concentrations

Ingestion of BR for six subsequent days resulted in elevated nitrate concentrations when compared to PLA, in both plasma (**Figure. 4.1A**) and saliva (**Figure. 4.1C**) (both $P<0.001$). Similarly, nitrite concentrations were higher following BR vs PLA supplementation in both plasma (632±66 nM vs. 186±13 nM; $P<0.001$; **Figure. 4.1B**) and saliva (2,882±519 µM vs 375±54 µM; $P<0.001$; **Figure. 4.1D**).

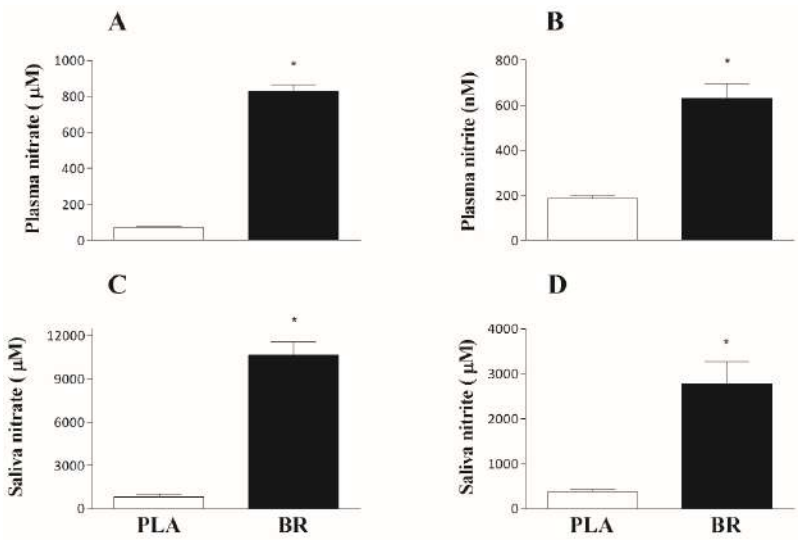

Figure 4.1. Mean plasma nitrate (A) and nitrite (B), and saliva nitrate (C) and nitrite (D) concentrations ~2.5 h after ingestion of the final supplemental bolus for the placebo (PLA) and the six day nitrate-rich beetroot juice (BR) intervention. Data are means ± SEM (n= 32). * BR significantly different from PLA ($P<0.001$).

Yo-Yo IR1 test

High-intensity intermittent running performance as assessed by the Yo-Yo IR1 significantly improved following BR ingestion (1,623±48 m) when compared to PLA (1,574±47 m; *P*=0.027; **Figure. 4.2A**). The average improvement in distance covered during the test was 3.4±1.3%, with a Cohen's d_z of 0.41. Of the 32 subjects assessed, 18 showed an improved performance during the BR trial vs. the PLA trial (+9±5%), 10 had a slightly worse performance (-5±3%) and 4 showed no difference between trials. Although peak heart rate did not differ between trials (*P*=0.16; **Table 4.1**), average heart rate during the Yo-Yo test was lower in the BR trial when compared to PLA (*P*=0.014; Table 4.1).

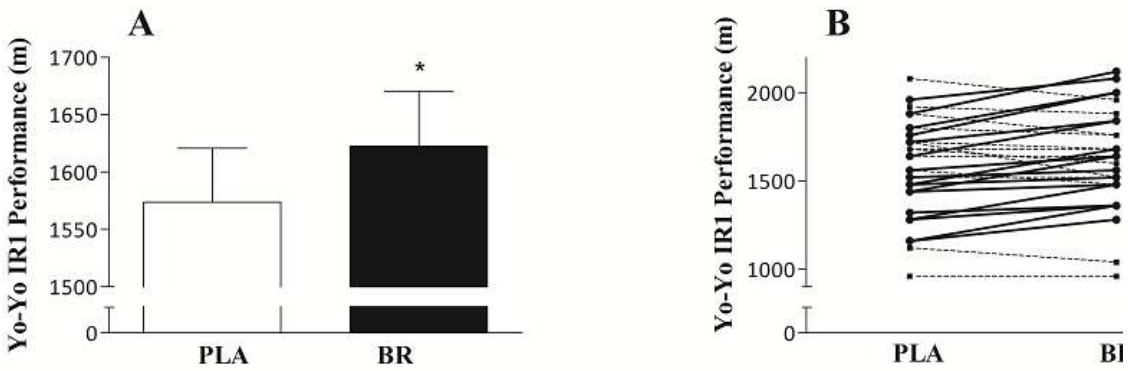

Figure 4.2. Mean distance covered during the Yo-Yo IR 1 test (A), and the individual response (B) following six days of placebo (PLA) and 6 days of nitrate-rich beetroot juice (BR) ingestion. *Distance covered following BR was significantly greater (3.4%) than that covered following PLA ingestion (*P*=0.027). Solid lines (-) indicate subjects that showed an improved performance following BR ingestion (*n*=18). Dashed lines (--) indicate subjects that showed a similar performance (*n*=4) following BR or PLA ingestion, or subjects that showed a worse (*n*=10) performance following BR ingestion.

GI and Borg Score

Subjects tolerated the interventional drinks well and GI discomfort did not differ between interventions. Only two participants reported a bloated stomach during the PLA trial, and one during the BR trial, while flatulence was reported by two participants during the PLA and two participants during the BR trial. Ratings of perceived exertion as determined with the Borg scale were also not different between interventions (*P*=0.23; Table 4.1).

Table 4.1. Heart rate data and Rate of perceived exertion

	PLA	**BR**
Mean heart rate (bpm)	175±2	172±2*
30-sec max heart rate (bpm)	191±1	190±1
RPE (Borg score)	17.6±0.3	17.3±0.4

All values are means ± SEM (n=25 for HR and n=32 for RPE). * Significantly different from PLA (*P*<0.05).

Correlation analyses

Despite the substantial elevations in plasma and saliva concentrations following BR ingestion, no significant correlations were found between plasma and saliva nitrate (r = 0.076, P=0.697) or plasma and saliva nitrite (r = 0.264, P=0.144) concentrations. In addition, no associations were observed between (differences in) plasma or saliva concentrations on the one hand, and the (differences in) distance covered, or heart rate variables on the other hand (all $r \leq 0.296$; all $P \geq 0.092$).

Discussion

The current study demonstrates that six days of nitrate-rich beetroot juice supplementation improves high-intensity intermittent type exercise performance in trained soccer players. The improvements in intermittent-type exercise performance were accompanied by a lower mean heart rate during the high-intensity intermittent running test, and were preceded by increases in both plasma and saliva nitrate and nitrite concentrations.

Nitrate related research in the past years has primarily focused on establishing the effects of nitrate supplementation on endurance type exercise performance. While improvements in exercise capacity(4, 8, 23) and exercise performance (5, 6) have indeed been observed in endurance athletes, recent literature suggests possible performance benefits of nitrate ingestion in more high-intensity and intermittent type sports and activities (16, 24). Extending on previous observations in recreationally active team-sport players (17), the present study assessed the effects of a multiday supplementation protocol with nitrate-rich beetroot juice on high-intensity intermittent type exercise performance in a large sample of trained soccer players.

We found that six days of BR supplementation elevated nitrate and nitrite concentrations in both plasma and saliva (Figure. 4.1). The observed 11-fold increase in plasma nitrate and 3-fold increase in plasma nitrite concentrations are in line with previous observations where a similar nitrate dose was administered(18, 24). In addition to the changes in plasma concentrations, the current findings suggest that saliva samples might represent a (less invasive) alternative to assess the postprandial response to beetroot juice ingestion. Salivary nitrate and nitrite concentrations were respectively 13-fold and 7-fold higher following BR ingestion when compared to PLA (Figure 4.1C-D). However, no correlations were observed between plasma concentrations and saliva concentrations. As such, it seems that saliva samples may be used as a means to assess compliance to nitrate supplementation and to confirm the endogenous reduction of nitrate into nitrite. Nevertheless, analysis of salivary nitrate and nitrite does not seem to represent a valid

surrogate for quantitative changes in plasma nitrate or nitrite concentrations.

In addition to changes in nitrate and nitrite concentrations, the six day BR supplementation protocol also resulted in quantifiable improvements in high-intensity intermittent running performance in the soccer players. We observed a 3.4% increase in intermittent-type exercise performance on the Yo-Yo IR1 test (Cohen's d_z: 0.41; Figure 4.2A). This is in line with a previous report of improvements in high speed running performance in recreationally active team sport players following a multiday BR supplementation regimen (17). Although the exact mode of action explaining this effect is still unclear, animal studies have shown that nitrate supplementation can increase blood flow (10), and enhance contractile function in type II muscle fibers (11). There is some suggestion that these adaptations might be responsible for the improved performance observed during high intensity/intermittent type exercise in which type II fibers are heavily recruited (25). Interestingly, while such cellular changes have been proposed to only occur following a multiday supplementation regimen (10, 11, 26), two studies from the same laboratory observed improvements in high-intensity intermittent type exercise performance following both an acute high dose BR supplementation protocol (~29 mmol within 36 h; 4.2% improvement)(16), as well as following a five day BR supplementation approach with a lower daily dose of nitrate (6.4 mmol/day; 3.9% improvement)(17). The use of a multiday protocol would seem preferred as it likely allows sufficient time for (some of) the suggested cellular adaptations to occur, that might drive the ergogenic effects of nitrate (10, 11). Furthermore, it is believed that trained subjects may require a different nitrate supplementation strategy (i.e. higher dose and/or for a longer period) to elicit beneficial performance effects in comparison to recreational athletes (9, 27, 28). The current study therefore assessed the ergogenic effect of a conventional 6-day supplementation protocol with BR (12.9 mmol/day nitrate) in a homogenous sample of trained soccer players. Performance on the Yo-Yo IR1 test was on average ~15% higher when compared to the recreational subjects included in the recent study from Thompson *et al*. (17). Nonetheless, we observed a 3.4% improvement in high-intensity intermittent running performance, suggesting that a six day BR supplementation protocol represents a practical and effective regimen for trained soccer players to improve their performance.

Clearly, such a performance benefit should be attained without any major negative side effects. In line with previous work (18), only very mild GI discomfort was reported in a few subjects during the current study, supporting the non-adverse use of beetroot juice in relatively short-term interventions. Furthermore, as recently reviewed by Bryan and Ivy (29), there is currently no clear indication of adverse health risks accompanying high

nitrate intakes for a prolonged period of time. At present, though any potential risks always need to be carefully considered, the established benefits of nitrate, which may be even more pronounced when consuming nitrate through 'natural' nitrate-rich vegetable sources (18, 19), seem to outweigh the risks (29).

Intriguingly, and in contrast to previous studies in team sport players, we observed that ingestion of BR for six consecutive days also had an effect on heart rate during the high-intensity intermittent running test (Table 4.1). While no changes were observed in peak heart rate, mean heart rate following BR ingestion was lower during the Yo-Yo IR1 than following PLA ingestion. To the best of our knowledge, the current findings are the first evidence of changes in heart rate following nitrate ingestion in young healthy athletes. Whether the decrease in mean heart rate is related to the improved exercise performance is unclear, as the only available literature describing effects of inorganic nitrate-nitrite on heart rate are from heart failure patients (30, 31). Borlaug and colleagues showed that a nitrite infusion protocol in heart failure patients increased cardiac output during exercise (30). The observed increase in stroke volume was suggested to be explained by improved contractility of the left ventricle. Although it is currently unclear whether nitrate and/or nitrite ingestion can similarly increase cardiac contractility in healthy individuals, such an effect could explain the decrease in heart rate observed in our study; i.e., allowing the same cardiac output with increased stroke volume, but lower heart rate. Interestingly, a recent study in rodents also showed increased cardiac contractility following nitrate ingestion, most likely as a result of enhanced expression of calcium handling proteins (32). As nitrate ingestion has also been shown to enhance expression of calcium handling proteins in type II skeletal muscle fibers (11), such an explanation would fit with both the observed increase in intermittent-type exercise performance, and the lower mean heart rate in the current study.

Although nitrate supplementation increased plasma and saliva nitrate and nitrite concentrations, improved exercise performance, and reduced heart rate, no correlations were observed between any of these parameters. Only a limited number of studies have been able to show correlations between plasma concentrations and subsequent performance benefits (2, 24, 27). In the present study, only a single sample of saliva and plasma was collected ~30 min prior to the exercise test. It could be suggested that a time point closer to, or even during the exercise test may have revealed a relation between plasma concentrations and changes in performance. Despite the fact that all subjects showed substantially increased plasma and saliva nitrate and nitrite concentrations, not all subjects showed improvements in performance (Figure 4.2B). It is unclear what the

exact explanation is for this lack of effect, although it seems likely that the large day-to-day variability inherent to the Yo-Yo test played a role (33)(Figure 4.2B). Taking this variability into account, the inclusion of a large sample of trained soccer players allowed us to show a significant and relevant improvement in Yo-Yo test performance following BR ingestion. Importantly, Yo-Yo IR1 performance has been described to strongly correlate with the ability to perform high speed running and sprinting activities throughout a soccer match (15). As such, our findings suggest that nitrate supplementation could represent an effective nutritional strategy to improve exercise performance in soccer players, especially towards the end of the match when sprint intensity/frequency has been shown to decrease significantly due to fatigue (34). Although in general, day-to-day variation in exercise performance tests combined with small sample sizes make it difficult to study potential ergogenic benefits in highly-trained athletes, future work should be undertaken to establish whether these performance improvements in high-intensity intermittent-type exercise in trained soccer players can also be translated towards the elite level.

Conclusion

Based on the present findings in a large sample of trained soccer players, we conclude that six days of nitrate-rich beetroot juice ingestion improves high-intensity intermittent type exercise performance.

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Chapter 5

Sodium nitrate ingestion increases skeletal muscle nitrate content in humans

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Abstract

Background: Nitrate (NO_3^-) ingestion has been shown to have vasoactive and ergogenic effects that have been attributed to increased nitric oxide (NO) production. Recent observations in rodents suggest skeletal muscle tissue to serve as an endogenous NO_3^- "reservoir". The current study determined NO_3^- contents in human skeletal muscle tissue in a post-absorptive state, and following ingestion of a sodium nitrate bolus ($NaNO_3$).

Methods: Seventeen male, type 2 diabetes patients (age 72±1 y; BMI 26.5±0.5 $m \cdot kg^{-2}$) were randomized to ingest a dose of $NaNO_3$ (NIT; 9.3 mg NO_3^- per kg bodyweight) or placebo (PLA; 8.8 mg NaCl per kg bodyweight). Blood and muscle biopsy samples were taken before and up to 7 h following NO_3^- or placebo ingestion to assess NO_3^- (and plasma nitrite (NO_2^-)) concentrations. Additionally, basal plasma and muscle NO_3^- concentrations were assessed in 10 healthy young (CON-Y: age 21±1 y) and 10 healthy older (CON-O: age 75±1 y) control subjects.

Results: In all groups, baseline NO_3^- concentrations were higher in muscle (NIT: 57±7, PLA: 61±7, CON-Y: 80±10, CON-O: 54±6 $\mu mol \cdot L^{-1}$) than in plasma (NIT: 35±3, PLA: 32±3, CON-Y: 38±3, CON-O: 33±3 $\mu mol \cdot L^{-1}$; $P \leq 0.011$). Ingestion of $NaNO_3$ resulted in a sustained increase in plasma NO_3^-, plasma NO_2^-, and muscle NO_3^- concentrations (up to 185±25 $\mu mol \cdot L^{-1}$) in the NIT group (time effect $P<0.001$), when compared with PLA (treatment effect $P<0.05$).

Conclusion: In conclusion, basal NO_3^- concentrations are substantially higher in human skeletal muscle tissue when compared with plasma. Ingestion of a bolus of dietary NO_3^- increases both plasma and muscle NO_3^- contents in humans.

Introduction

In the past 2 decades, nitrate (NO_3^-) and nitrite (NO_2^-) have evolved from only being viewed as inactive 'waste' products of the nitric oxide synthase (NOS)-dependent nitric oxide (NO) metabolism, to also being considered precursors of NO through a reversed pathway (1). In this pathway that is strongly facilitated by hypoxia and a low pH, NO_3^- is first reduced back into NO_2^-, before finally stimulating NOS-independent NO synthesis (2). In recent years, various studies have shown that ingestion of nitrate-rich foods, primarily red beetroot and green-leafy vegetables, effectively increases plasma NO_3^- and NO_2^- concentrations (3-6). Elevated concentrations of these anions have in turn been associated with several pharmacodynamic effects that have been attributed to an increased NO-bioavailability, such as a decrease in blood pressure and a reduction in oxygen cost during submaximal exercise (3, 7-10).

Exploration of the various pharmacodynamic effects of dietary NO_3^- has also resulted in a better understanding of the pharmacokinetics. For example, we now know that plasma NO_3^- concentrations rapidly increase after the ingestion of dietary NO_3^-, peaking at ~0.5-2 h post-ingestion (3). This is shortly followed by an increase in plasma NO_2^- concentrations, with peak levels observed ~2-3 h following NO_3^- ingestion (7). The increase in plasma NO_2^- concentration has been described to be the result of the entero-salivary circulation of NO_3^-, in which ingested NO_3^- that is taken up into the circulation, is concentrated in the salivary glands and is subsequently (partly) reduced to NO_2^- by commensal bacteria in the oral cavity (11). The newly formed NO_2^- is then swallowed, before subsequently being taken up into the systemic circulation (2). It has now become common practice to present the *in vivo* metabolism of dietary NO_3^- with plasma concentrations of NO_3^- and NO_2^-, and to associate these changes with various pharmacodynamic effects that follow (10, 12, 13).

Interestingly, a recent study measured NO_3^- concentrations in rodent muscle, and showed that basal muscle NO_3^- concentrations were higher than the concentrations measured in blood and liver (14). Based on these findings, the authors suggested that muscle tissue may serve as a "reservoir" for NO_3^- *in vivo* (14). It could be speculated that the intramuscular storage of NO_3^- can be used to rapidly increase NO-bioavailability through the NO_3^- - NO_2^- - NO pathway, especially during situations in which the NOS-pathway might not suffice (i.e. during hypoxia) (2). Such a function in rodents might also suggest the need for a NO_3^- "reservoir" in human skeletal muscle tissue to rapidly increase NO-bioavailability during hypoxia, or when suffering from a pathological condition with impaired NOS functioning (15). However, it is currently unknown whether NO_3^- is also present in human muscle.

In light of the growing interest in the pharmacokinetics and pharmacodynamics of dietary NO_3^-, we therefore investigated whether NO_3^- is present in skeletal muscle tissue under basal conditions, and whether the ingestion of dietary NO_3^- increases both plasma and skeletal muscle NO_3^- and NO_2^- concentrations *in vivo* in humans. We measured both plasma and skeletal muscle tissue NO_3^- and NO_2^- concentrations at baseline and up to 7 hours after ingestion of either a single bolus of dietary NO_3^- (9.3 mg NO_3^- per kg bodyweight) or NaCl (8.8 mg per kg bodyweight) in male volunteers. We hypothesized that muscle NO_3^- concentrations would be higher than NO_3^- concentrations in plasma, and that ingestion of a single dietary NO_3^- bolus would increase NO_3^- concentrations in both plasma and skeletal muscle tissue.

Methods

Subjects

Plasma and skeletal muscle tissue samples of seventeen male type 2 diabetes patients (age 72±1 y; body mass index (BMI) 26.5±0.5 $m \cdot kg^{-2}$; glycated Hemoglobin (HbA_{1C}) 7.2±0.2%) were included for the primary analyses of the current study. The samples were collected during a larger project on muscle protein metabolism (16) in 24 older type 2 diabetes mellitus patients (T2DM). The T2DM patients in that study were randomly assigned to ingest a single dose of protein with either sodium chloride (PLA; NaCl) or sodium nitrate (NIT; $NaNO_3$) following a resting, basal period. As a result of the analyses of muscle protein metabolism for the main research aim of that study (16), sufficient plasma and muscle tissue was only available from 17 of the 24 T2DM patients to measure basal and post-ingestion NO_3^- and NO_2^- concentrations for the current study aim. Furthermore, to determine whether factors such as age and disease state might have modulated basal NO_3^- metabolism in the T2DM patients, we also included basal plasma and muscle samples in the analyses, from 10 healthy young male controls (CON-Y; age 21±1 y; BMI 22±0.6 $m \cdot kg^{-2}$) and 10 healthy older male controls (CON-O; age 75±1 y; BMI 24±1.2 $m \cdot kg^{-2}$) from a second project (17). Subjects' characteristics are presented in **Table 5.1**. Participants from both projects were informed about the nature and possible risks of the experimental procedures prior to obtaining written informed consent. The trials were conducted between August 2011 and July 2012, at Maastricht University Medical Centre, the Netherlands. The studies were approved by the Medical Ethical Committee of the Maastricht University Medical Centre, the Netherlands, and were registered as NCT01473576 (T2DM groups) and NCT01576848 (CON groups), and conformed to standards for the use of human subjects in research as outlined in the latest version of the Declaration of Helsinki.

Table 5.1. Subjects' characteristics

	PLA	NIT	CON-Y	CON-O
Age (y)	72±1	72±1	21±1*	75±1
Weight (kg)	79.3±2.0	82.3±2.5	73.9±3.4	72.1±4.8
BMI ($kg \cdot m^{-2}$)	26.3±0.6	27.0±0.8	22.2±0.6#	24.0±1.2
Lean body mass (kg)	59.8±1.1	61.2±2.1	59.6±1.9	55.2±2.9
Basal plasma glucose ($mmol \cdot L^{-1}$)	9.4±1.1	9.4±0.6	5.2±0.09	5.4±0.08
Basal plasma insulin ($mU \cdot L^{-1}$)	15.7±2.3	14.9±0.7	7.3±1.3#	6.0±1.3#
HbA_{1C} (%)	7.1±0.3	7.5±0.3	5.2±0.1#	5.7±0.1#

Values are mean±SEM. PLA= placebo group (*n*=9). NIT= sodium nitrate group (*n*=8). CON-Y = young controls (*n*=10). CON-O = elderly controls (*n*=10). HbA_{1C}: glycosylated hemoglobin. Data were analyzed using a one-way ANOVA with Bonferroni corrected post-hoc testing. * Indicates a significant difference when compared with PLA, NIT and CON-O ($P<0.001$). # Indicates a significant difference when compared with PLA and NIT ($P<0.01$).

Experimental protocol

Apart from the NO_3^- and NaCl supplemented participants being diagnosed with type 2 diabetes, all subjects were deemed healthy based on their responses to a medical questionnaire and screening results. All subjects were instructed to refrain from strenuous physical activity and to maintain their diet as constant as possible for 2 d prior to the test day. On the evening before the trial, all subjects consumed a standardized meal as described previously (16, 17).

At 08:00 AM, following an overnight fast, subjects arrived at the laboratory by car or public transport. A Teflon catheter was inserted into the dorsal hand vein and was placed in a hot-box (60˚C) for arterialized blood sampling. After baseline blood and muscle collection, (t = 0 min), the T2DM patients ingested a single bolus of $NaNO_3$ (12.8 $mg \cdot kg^{-1}$ body weight, which provided 9.3 mg or 0.15 mmol NO_3^- per kg bodyweight; BASF, Ludwigshafen, Germany) or an equimolar amount of NaCl (which provided 8.8 mg NaCl per kg bodyweight; Glacia British salt, United Kingdom) dissolved in 250 mL of water, as described previously (16). The administered dose was based on a previous dose-response study in healthy subjects that concluded that ~8.4 mmol (or 0.12 mmol per kg bodyweight for a 70 kg weighing adult) of dietary NO_3^- was effective in increasing plasma NO_3^- and NO_2^- concentrations, as well as decreasing blood pressure and improving exercise performance (7). However, to account for the possibly attenuated response to dietary NO_3^- in T2DM

patients as a result of their disease, the dose in the current investigation was increased slightly to provide 0.15 mmol of NO_3^- per kg bodyweight. Blood samples were collected at *t*= 30, 60, 90, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, 360, 390, and 420 min relative to the ingestion of $NaNO_3$ or NaCl. Blood samples were collected in EDTA-containing tubes and centrifuged at 1000*g* at 4˚C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at -80˚C. Muscle biopsies were collected at *t*= 0, 120, 240, and 420 min relative to the ingestion of $NaNO_3$ or NaCl. Only the basal plasma and skeletal muscle samples of the T2DM were compared with the basal samples of the young and older controls. Muscle biopsies were collected from the middle region of the *M. vastus lateralis* (15 cm above the patella) using the Bergström needle technique (18). In short, following local anesthesia (2% xylocaine), the procedure consisted of a small incision being made in skin and fascia after which a Bergström muscle biopsy needle modified for manual vacuum was introduced in the muscle, and several small muscle samples ('clips') were collected. All biopsy samples were placed on a petri-dish and freed from any visible adipose tissue and blood using a curved thumb-dissecting-forceps and a scalpel, before being subsequently frozen in liquid nitrogen (within ~1 min after collection), and stored at -80˚C until analysis.

Nitrate and nitrite analyses

Both plasma and muscle NO_3^- concentration and plasma NO_2^- concentration were quantified through their initial reduction to NO gas using the gas-phase chemiluminescence technique (19). For determination of NO_3^- concentration, plasma samples were first deproteinized in an aqueous solution of zinc sulphate (10% w/v) and 1 M sodium hydroxide, prior to the reduction of NO_3^- to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v). For NO_2- concentrations, undiluted plasma was injected into a glass purge vessel containing 5 mL glacial acetic acid and 1 mL NaI solution. To assure a valid determination of muscle NO_3^- concentration, a piece of wet muscle (~40 mg) was first freeze-dried, followed by removing collagen, blood, and other non-muscle tissue from the freeze-dried sample under a dissecting microscope. The freeze-dried muscle was then weighed (~9 mg) and homogenized in 35 volumes (7 × dry weight × wet/dry ratio) of ice-cold 2% perchloric acid (PCA). Samples were incubated on ice for 10 min. After centrifugation of the samples at 1000*g* at 4˚C for 4 min, the supernatant was collected for subsequent determination of NO_3^- concentrations by gas-phase chemiluminescence. Determination of NO_2^- concentration in supernatants of muscle tissue was not possible, as the concentrations remained below the detection limit of 2 $nmol \cdot g^{-1}$ dry weight muscle.

Quantification of NO after the reduction of NO_3^- and NO_2^- through chemiluminescence was achieved by the detection of light emitted during the production of nitrogen dioxide formed upon the reaction of NO with ozone. Luminescence was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyzer (Sievers NOA 280i, Analytix Ltd, Durham, UK). The concentration of NO_2^- was determined by plotting signal (mV) area against a calibration plot of 25 nM to 1 µM sodium nitrite. The concentration of NO_3^- was determined by plotting signal (mV) area against a calibration plot of 100 nM to 10 µM sodium nitrate.

Statistics

All data are expressed as mean±SEM. Differences in basal values, peak values, and time to peak between groups were determined using an unpaired, two-tailed Student's t-test within the T2DM patients, and using one-way ANOVA between T2DM patients and healthy young and old controls with Bonferroni corrected post-hoc testing where appropriate. In the T2DM patients, repeated measures ANOVA, with time as within-subject factor and treatment as between-subject factor, was used to compare differences over time in plasma and muscle NO_3^- concentrations, and plasma NO_2^- concentrations between groups. In case of significant interaction between time and treatment, separate analyses were performed to locate differences between treatments (two-tailed Student's t-test per time point) and time (repeated measures ANOVA per treatment group with Bonferroni corrected post-hoc testing where appropriate). Repeated measures ANOVA was also performed to assess between tissue (plasma and muscle) differences in the T2DM groups, with both time and tissue as within-subject factor. Muscle NO_3^- concentration values were first transformed from $nmol \cdot g^{-1}$ to $\mu mol \cdot L^{-1}$ to allow for proper comparison with plasma values. In case of a significant interaction between time and tissue, separate analyses were performed to locate differences between plasma and muscle NO_3^- concentration (paired samples t-test per time point). A paired samples t-test was also used to assess differences between plasma and muscle NO_3^- in the young and elderly controls. Pearson correlation coefficients were calculated to determine whether baseline concentrations of NO_3^- or NO_2^-, or changes in these concentrations were correlated between plasma and skeletal muscle tissue. For all analyses, statistical significance was set at $P<0.05$. All calculations were performed using SPSS Statistics (version 21, IBM Corp., Armonk, NY, USA).

Results

Plasma and muscle data in T2DM patients

Plasma NO_3^- concentrations are presented in **Figure 5.1**. There were no baseline differences in plasma NO_3^- concentrations between treatments ($P=0.47$). A significant time x treatment interaction ($P<0.001$) showed that plasma NO_3^- concentrations increased

following $NaNO_3$ ingestion (time effect $P<0.001$), and remained elevated above baseline levels at all time points ($P<0.001$) in the NIT group. Small but significant increases in plasma NO_3^- concentrations were also observed in the PLA group (time effect $P=0.003$), but plasma NO_3^- concentrations remained ~12 fold higher in the NIT when compared with the PLA group (treatment effect $P<0.001$). Peak plasma NO_3^- concentrations averaged 660±33 µmol·L^{-1} (at t= 64±4 min following $NaNO_3$ ingestion) in the NIT group and 39±3 µmol·L^{-1} (at t= 162±26 min) in the PLA group (Peak value: $P<0.001$; Time to peak: $P<0.01$).

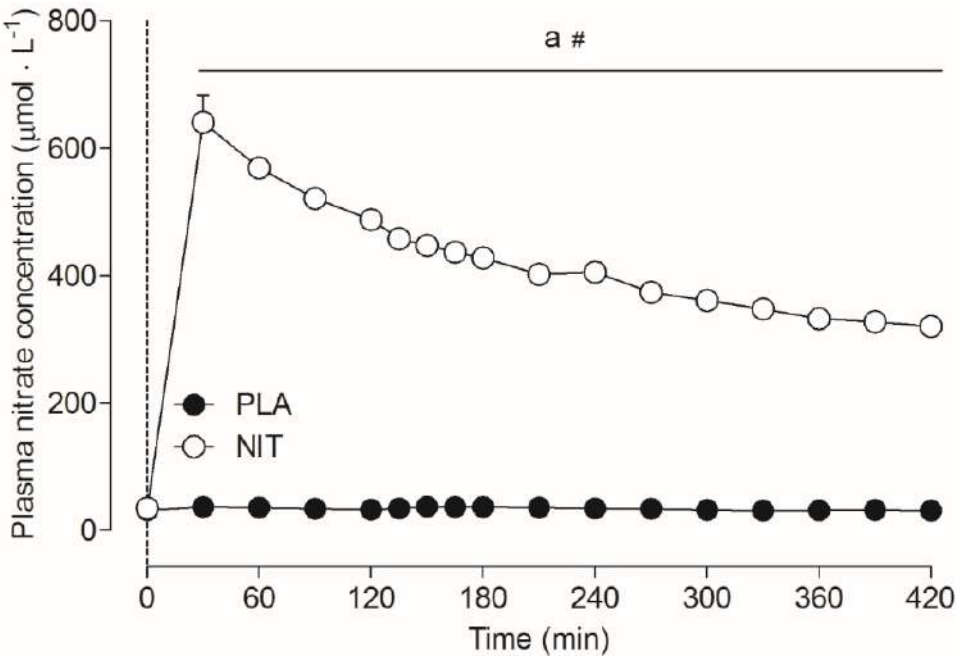

Figure 5.1. Mean (±SEM) plasma NO_3^- concentrations (µmol·L^{-1}) of type 2 diabetes patients following ingestion of a single dose of sodium nitrate (NIT; $NaNO_3$, n = 8) or sodium chloride (PLA; NaCl, n = 9). The dotted line indicates the ingestion of $NaNO_3$ or NaCl. Data were analyzed with repeated measures (time x treatment) ANOVA. Time effect $P<0.001$, treatment effect $P<0.001$, time x treatment $P<0.001$. [a] Significant difference compared with baseline values, $P<0.001$. [#] Significant difference compared with PLA, $P<0.001$.

Plasma NO_2^- concentrations are presented in **Figure 5.2**. Plasma NO_2^- concentrations did not differ between treatments at baseline ($P=0.42$). In line with plasma NO_3^- concentrations, a significant time x treatment interaction was observed for plasma NO_2^- concentrations following $NaNO_3$ ingestion ($P=0.047$). Plasma NO_2^- concentrations increased in the NIT group only (time effect $P=0.037$), with no changes being observed in the PLA group ($P=0.17$). Plasma NO_2^- concentrations tended to be higher in the NIT when compared with the PLA group between t= 90 and 150 min and at t= 210 min (all $P<0.1$). Peak plasma NO_2^- concentrations averaged 852±212 nmol·L^{-1} at t= 178±20 min in NIT and 427±53 nmol·L^{-1} at t= 262±28 min in PLA (Peak value: $P=0.058$; Time to peak: $P=0.032$).

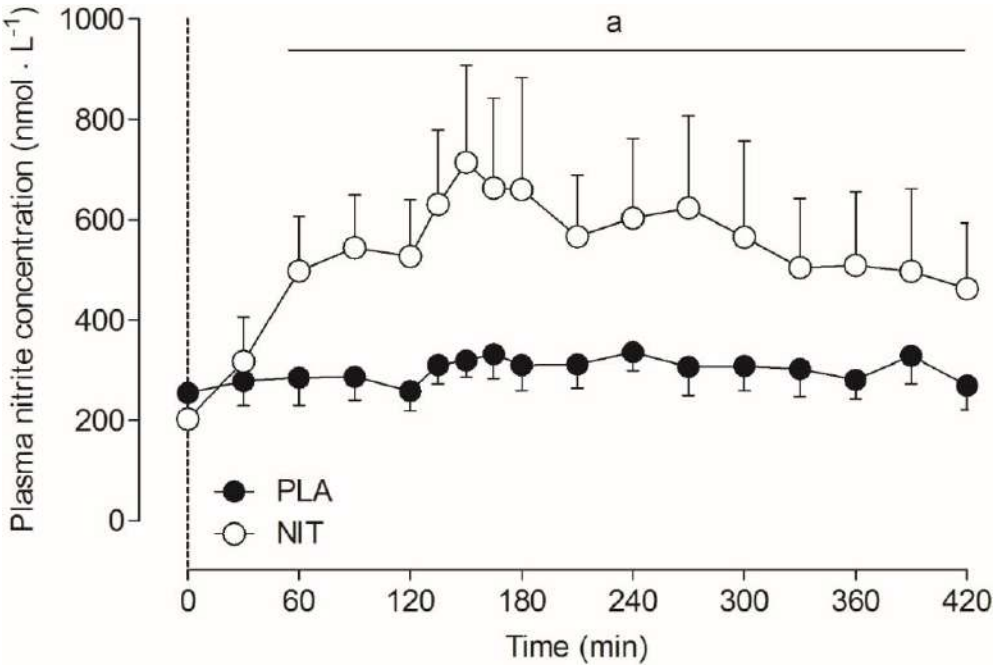

Figure 5.2. Mean (±SEM) plasma NO_2^- concentrations (nmol·L^{-1}) of type 2 diabetes patients following ingestion of a single dose of sodium nitrate (NIT; $NaNO_3$, *n* = 8) or sodium chloride (PLA; NaCl, *n* = 9). The dotted line indicates the ingestion of $NaNO_3$ or NaCl. Data were analyzed with repeated measures (time x treatment) ANOVA. Time effect *P*<0.01 and time x treatment *P*<0.05. [a] Significant difference compared with baseline values, *P*<0.05;

Skeletal muscle tissue NO_3^- concentrations are presented in **Figure 5.3.** Skeletal muscle NO_3^- concentrations at baseline averaged 61±7 and 57±7 nmol·g^{-1} wet weight (ww) in the PLA and NIT group, respectively, with no differences between treatment groups (*P*=0.7). Also in muscle, a significant time x treatment interaction was observed (*P*<0.001). Following $NaNO_3$ ingestion, muscle NO_3^- concentrations increased (time effect *P*<0.001), and remained well above baseline values at all time points (*P*<0.05) in the NIT group. No changes were observed in muscle NO_3^- concentrations in the PLA group (time effect *P*=0.6). Skeletal muscle NO_3^- concentrations in the NIT group were significantly higher when compared with the PLA group at all time points following nitrate ingestion (*P*≤0.001). Muscle NO_3^- concentrations peaked at 217±24 nmol·g^{-1} ww in the NIT group at *t*= 75±22 min following $NaNO_3$ ingestion and 71±6 nmol·g^{-1} ww at *t*= 47±44 min in the PLA group (Peak values: *P*<0.001; Time to peak: *P*=0.59).

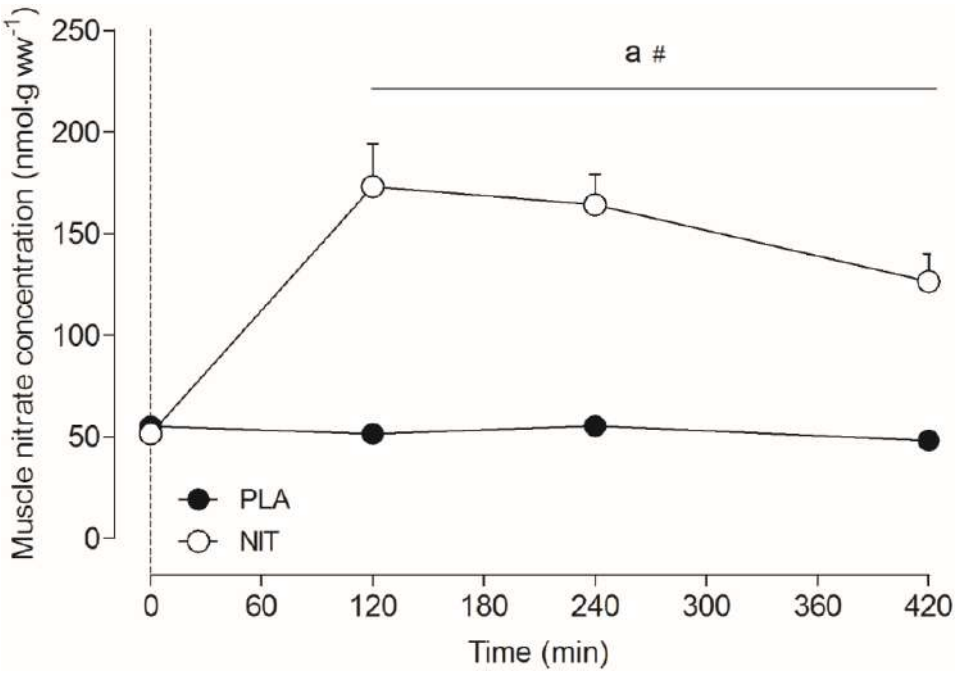

Figure 5.3. Mean (±SEM) skeletal muscle NO_3^- concentrations (nmol·g^{-1} wet weight) of type 2 diabetes patients following ingestion of a single dose of sodium nitrate (NIT; $NaNO_3$, *n* = 8) or sodium chloride (PLA; NaCl, *n* = 9). The dotted line indicates the ingestion of $NaNO_3$ or NaCl. Data were analyzed with repeated measures (time x treatment) ANOVA. Time effect *P*<0.001, treatment effect *P*<0.001, time x treatment *P*<0.001. [a] Significant difference compared with baseline values, *P*<0.05. [#] Significant difference compared with PLA, *P*<0.001.

Type 2 diabetes patients versus healthy controls

To determine whether factors such as age and disease state might have modulated basal NO_3- metabolism in the T2DM patients, we also assessed basal plasma and muscle NO_3- concentrations in 10 healthy young males (CON-Y) and 10 healthy elderly males (CON-O). Plasma and muscle NO_3^- are presented in **Figure 5.4**. Basal plasma NO_3^- concentrations did not differ between CON-Y and CON-O, and were similar to the concentrations observed in T2DM patients (difference between groups *P*=0.41). For post-absorptive muscle NO_3^- concentrations, a significant difference between the three groups was observed (*P*=0.035). Bonferroni-corrected post-hoc tests showed lower values in the CON-O group than in the CON-Y group (*P*=0.049), and a trend towards lower muscle NO_3^- concentrations in T2DM patients when compared to CON-Y subjects (*P*=0.09). The muscle NO_3^- concentrations did not differ between T2DM patients and their age-matched CON-O (*P*=1.0; Figure 5.4).

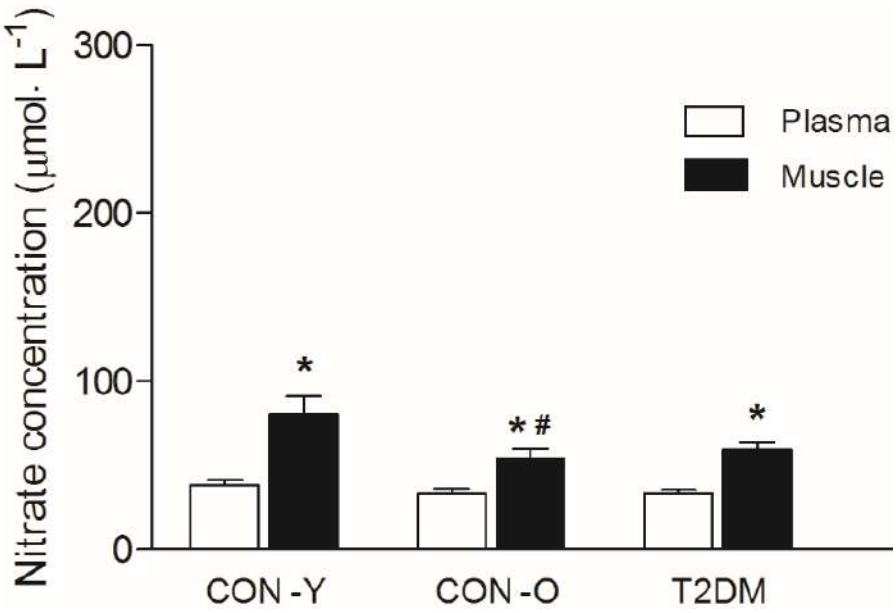

Figure 5.4. Mean (±SEM) skeletal muscle NO_3^- concentrations (µmol·L^{-1}) and plasma NO_3^- concentrations (µmol·L^{-1}) of type 2 diabetes patients (T2DM, *n* = 17), healthy young controls (CON-Y, *n* = 10) and healthy older controls (CON-O, *n* = 10) under basal conditions. Data were analyzed with paired samples t-test (muscle vs plasma, within groups) and one-way ANOVA (between groups).; * Significant difference compared with plasma, *P*<0.05. # Significant difference compared with muscle NO_3^- concentrations in CON-Y, *P*<0.05. A trend (*P*=0.09) was observed for a difference in muscle NO_3^- concentrations between T2DM and CON-Y).

Muscle versus plasma

Comparison of NO_3^- concentrations in skeletal muscle tissue and plasma under basal conditions revealed that muscle NO_3^- concentrations were higher than plasma NO_3^- concentrations in all groups; T2DM patients (*P*<0.001), CON-Y (*P*=0.001), and CON-O (*P*=0.011) (Figure 5.4). Ingestion of $NaNO_3$ by T2DM patients in the NIT group, resulted in plasma NO_3^- concentrations increasing up to mean values of ~500 µmol·L^{-1}, and muscle NO_3^- concentrations increasing up to ~200 µmol·L^{-1}; both plasma and muscle NO_3^- concentrations remained significantly elevated above baseline values throughout the 7 h test period that followed (*P*<0.001; **Figure 5.5**). The muscle NO_3- concentrations in the T2DM patients PLA group remained higher than plasma concentrations throughout the 7 h test period (*P*≤0.009; Figure 5.5). No significant correlations were observed between plasma and muscle NO_3^- concentrations in the T2DM patients or in the control groups (all *P*>0.15; *data not shown)*.

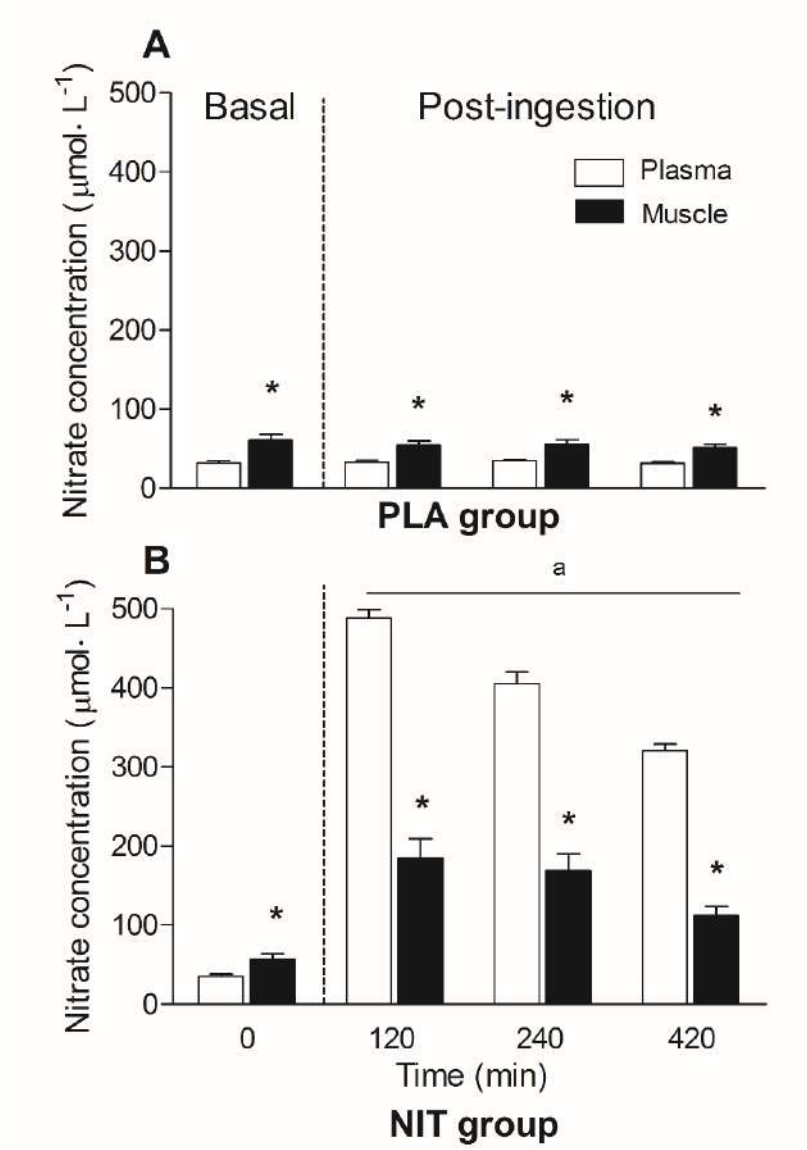

Figure 5.5. Mean (±SEM) skeletal muscle NO_3^- concentrations (µmol·L^{-1}) and plasma NO_3^- concentrations (µmol·L^{-1}) of type 2 diabetes patients following ingestion of a single dose of sodium chloride (PLA; NaCl, *n* = 9; A) or sodium nitrate (NIT; $NaNO_3$, *n* = 8; B). The dashed line indicates the ingestion of $NaNO_3$ or NaCl. Data were analyzed with repeated measures (time x tissue) and paired samples t-test (muscle vs plasma). [a] Significant difference compared with baseline values, *P*<0.005. * Significant difference compared with plasma, *P*<0.05.

Discussion

This study is the first to present basal NO_3^- concentrations in human skeletal muscle tissue of healthy males and T2DM patients, as well as the subsequent changes in plasma and muscle NO_3- concentrations of T2DM patients following dietary NO_3^- ingestion. We demonstrate that under basal conditions, NO_3^- concentrations are higher in human skeletal muscle than in plasma. The ingestion of dietary NO_3^- substantially increases NO_3^- concentrations in both plasma and skeletal muscle tissue.

The consumption of nitrate-rich foods has seen a strong increase in the past years due to

an accumulating body of evidence establishing the beneficial effects of dietary NO_3^- (3-5, 20, 21). Ingestion of NO_3^- through vegetable intake, such as red beetroot (juice) and spinach has been shown to have both vasoactive (3, 8, 10, 22-26) and ergogenic effects (8, 9, 27). Research has provided insight in the pharmacokinetics of dietary NO_3^- mainly by presenting changes in plasma NO_3^- and NO_2^- concentrations (3, 7). However, a recent animal study described measurements of NO_3^- concentration in skeletal muscle tissue in rodents, and suggested skeletal muscle to serve as an *in vivo* NO_3^- "reservoir" (14). Currently no literature is available of similar observations in humans.

In the present study, we assessed both plasma and muscle NO_3^- and NO_2^- concentrations before and after the ingestion of a bolus of $NaNO_3$. We observed that under basal conditions, NO_3^- was not only present in plasma, but also in skeletal muscle tissue of T2DM patients (Figure 5.1 and 5.3). In fact, NO_3^- concentrations in skeletal muscle tissue were observed to be even higher than plasma concentrations. Importantly, similar findings were observed in both young and older healthy controls, suggesting the existence of a NO_3^- "reservoir" in human skeletal muscle. No significant correlations were observed between plasma and muscle NO_3^- concentrations. The absence of such a correlation may indicate that NO_3^- homeostasis is regulated differently within the two compartments. While comparative data from humans is currently lacking, these findings seem to be in accordance with previous observations in rodent models (14, 28). Piknova *et al.* (14) showed that NO_3^- concentrations in rodent muscle were higher when compared to concentrations in blood. The authors suggested that skeletal muscle tissue may serve as an endogenous buffer of NO_3^- (and NO_2^-). The ~76% higher NO_3^- concentrations in skeletal muscle when compared with plasma in the present study implies a similar function in human skeletal muscle tissue.

In the current study we also assessed the impact of dietary NO_3^- ingestion on plasma and muscle NO_3^- concentrations of T2DM patients. We observed a ~19 fold increase in plasma NO_3^- concentrations ~64 min after ingesting 9.3 $mg \cdot kg^{-1}$ bodyweight NO_3^- in the NIT group, which is in line with previous findings from our group (3, 29) as well as others (30, 31) following ingestion of a comparable dose of NO_3^-. A slight increase in plasma NO_3^- concentration was also observed in the PLA group, which was likely a result of the tap water that was used for the interventional solutions that subjects consumed. We recently assessed the habitual intake of various dietary NO_3^- sources in Dutch athletes and found that tap water also contributed to the daily NO_3^- intake (32). Nonetheless, plasma NO_3^- concentrations in the PLA group remained significantly lower than what was observed in the NIT group (Figure 5.1), and no changes were observed in plasma NO_2^- concentrations

as a result of the trivial increase in plasma NO_3^- concentration (Figure 5.2). Furthermore a ~3 fold increase in muscle NO_3^- concentrations was only observed following $NaNO_3$ ingestion (Figure 5.3). While NO_3^- concentrations were higher in muscle when compared with plasma at baseline, plasma NO_3^- concentrations increased much stronger than muscle contents following dietary NO_3^- ingestion (Figure 5.5). Again, no correlations were observed between the increase in NO_3^- concentrations in plasma and muscle tissue. Although the larger increase in plasma NO_3^- concentrations might suggest a greater storage and/or redistribution capacity in plasma, the total compartment of skeletal muscle tissue (~30-40 kg) is clearly much greater than that of plasma (~5 L). As such, the substantial rise in NO_3^- content per unit of muscle tissue would support previous suggestions of muscle being the main reservoir for NO_3^- in the body (14).

Some caution is however warranted when interpreting these data, as postprandial muscle nitrate concentrations were only assessed in the T2DM patients. Type 2 diabetes has been shown to result in muscle microcirculatory dysfunction that may alter the pharmacokinetics of dietary NO_3^- (33). Interestingly, supplementation with dietary NO_3^- has recently been shown to increase limb perfusion during exercise in a chronic heart failure rodent model (15), as well as increase exercise tolerance in human peripheral artery disease patients (34). This seems to suggest a therapeutic potential for dietary NO_3^- to counteract microvascular impairments. Obviously, future studies will need to look further into the increase in plasma and muscle NO_3^- concentrations following dietary NO_3^- ingestion, as well as the subsequent pharmacodynamic effects, to confirm as well as elaborate on the present findings, e.g. studying different populations, using different dietary NO_3^- sources and potentially using stable isotope methodology to assess actual fluxes of NO_3^-.

In line with previous observations in healthy young subjects ingesting a similar dose (29), plasma NO_2^- concentrations in the T2DM patients increased ~3 fold following ingestion of dietary NO_3^-. However, the concentrations in skeletal muscle remained below the detection limit, which may have been caused by the limited amount of muscle tissue available form human biopsy samples (i.e., in contrast to the animal work by Piknova *et al.*(14)). Whether muscle NO_2^- concentrations would have been measurable in a more favorable milieu for NO_3^- to NO_2^- reduction is currently unclear. Future research aimed at quantifying NO metabolism in human muscle tissue might therefore need to combine measurements of NO_3^- and NO_2^- in muscle, with an intervention that stimulates reductase activity such as hypoxia or exercise (35, 36). Alternatively, researchers performing measurements without an intervention that promotes NO_3^- and NO_2^- reduction, should

consider including measurements of a downstream marker of increased NO bio-availability such as cGMP (6, 23). This might help gain more insight in the link between muscle NO_3^- and NO_2^- contents, muscle NO-synthesis, and the pharmacodynamics following dietary NO_3^- consumption.

Currently we can only speculate on the function of NO_3^- reserves in (human) skeletal muscle tissue. A recent study by Piknova and colleagues (28), described substantial decreases in muscle NO_3^- concentrations and transient increases in NO_2^- concentrations in muscle during exercise conditions in rodents. The authors suggested that the changes in NO_3^- and NO_2^- concentrations might be linked to the increased xanthine oxidoreductase (XOR) activity, which enhanced NO-bioavailability and as a result (partially) induced the functional hyperemia observed during exercise. Previous studies indeed concluded that XOR is capable of reducing NO_3^- into NO_2^- (36), and NO_2^- into NO (37), especially under hypoxic conditions. Interestingly, XOR has also been suggested to be present in human skeletal muscle tissue (35). Arguably, the availability of a NO_3^- / NO_2^- reducing enzyme in human skeletal muscle tissue might allow the 'buffered' NO_3^- to serve as a local NO precursor. Muscles with a large proportion of type II muscle fibers would seem to be the most likely location for such NO_3^- / NO_2^- reducing enzymes, considering the hypoxic milieu that strongly mediates anaerobic NO production (2). This might in turn also suggest the need for fiber-type-specific storage of NO_3^- to facilitate rapid availability of NO_3^- for conversion by reductase. Interestingly, a fiber-type-specific storage of NO_3^- would be in line with the increasing notion that NO_3^- might largely convey its effects through type II muscle fibers (38). This could also be a possible explanation for the differences observed in NO_3^- content in the current study between CON-Y and CON-O subjects, as the *vastus lateralis* muscle of healthy young males has been shown to have a significantly higher area percentage occupied by type II muscle fibers than the same muscle in elderly males (39). A tendency towards lower muscle NO_3^- concentrations in the elderly T2DM also seems to support an age-related decrease in NO_3^- storage capacity (Figure 5.4). The subtle difference in muscle NO_3^- concentrations between the elderly T2DM patients and CON-O, might be due to changes in muscle fiber-type composition suggested to result from T2DM, i.e. a substantial increase in the proportion of type II muscle fibers in the *m. vastus lateralis* of T2DM patients (40, 41). Although changes in systemic NO-bioavailability as a result of T2DM have been seen by some (42-44), plasma NO_3^- concentrations likely do not explain the observed differences in muscle NO_3^- concentrations, since we did not observe any differences in plasma NO_3^- concentrations between any of the groups (Figure 5.4). Future research will have to elucidate whether fiber-type specific buffering of NO_3^- indeed occurs in human skeletal muscle tissue, potentially explaining the current findings.

In conclusion, basal NO_3^- concentrations are much higher in human skeletal muscle tissue when compared with plasma concentrations. Furthermore, ingestion of dietary NO_3^- strongly elevates both plasma and skeletal muscle NO_3^- contents in humans. Dietary NO_3^- supplementation may represent a nutritional strategy to increase NO-bioavailability in conditions in which NOS-dependent NO synthesis and/or muscle microvascular function is impaired.

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Chapter 6

Blood flow restriction only increases myofibrillar protein synthesis with exercise

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Abstract

Purpose: Combining blood flow restriction (BFR) with exercise can stimulate skeletal muscle hypertrophy. Recent observations in an animal model suggest that BFR performed without exercise can also induce anabolic effects. We assessed the impact of BFR performed both with and without low-load resistance-type exercise (LLRE) on *in vivo* myofibrillar protein synthesis rates in young men.

Methods: Twenty healthy young men (age: 24±1 y, BMI: 22.9±0.6 kg/m^2) were randomly assigned to remain in resting condition (REST+/-BFR; *n*=10), or to perform LLRE (LLRE+/-BFR at 20%1RM; *n*=10), combined with two 5-min cycles of single leg BFR. Myofibrillar protein synthesis rates were assessed during a 5-h post-BFR period, by combining a primed continuous L-[ring-$^{13}C_6$]phenylalanine infusion with the collection of blood samples, and muscle biopsies from the BFR leg and the contralateral control leg. Phosphorylation status of anabolic signaling (mTOR pathway) and metabolic stress (ACC) related proteins, as well as mRNA expression of genes associated with skeletal muscle mass regulation were assessed in the collected muscle samples.

Results: Under resting conditions, no differences in anabolic signaling or myofibrillar protein synthesis rates were observed between REST+BFR and REST (0.044±0.004 vs 0.043±0.004 %/h, respectively; *P*=0.683). In contrast, LLRE+BFR increased myofibrillar protein synthesis rates by 10±5% compared with LLRE (0.048±0.005 vs 0.043±0.004 %/h, respectively; *P*=0.042). Furthermore, compared with LLRE, LLRE+BFR showed higher phosphorylation status of ACC and 4E-BP1 as well as elevated mRNA expression of MuRF1 (all *P*<0.05).

Conclusion: BFR does not increase myofibrillar protein synthesis rates in healthy young men under resting conditions. When combined with low-load resistance-type exercise, BFR increases post-exercise myofibrillar protein synthesis rates *in vivo* in humans.

Introduction

Muscle disuse due to reduced physical activity, immobilization or bed rest, has been shown to result in substantial decreases in muscle mass and strength (1, 2). These catabolic changes in skeletal muscle tissue have in turn been associated with functional disabilities and an increased risk of developing (chronic) metabolic impairments (3). A recent study from our laboratory for example, showed that 7 days of bed rest decreases quadriceps muscle cross-sectional area by 3.2%, and whole body insulin sensitivity by 29% in healthy young men (4). Such disuse-induced muscle loss and subsequent metabolic dysfunction underscore the need for effective interventional strategies to counteract these detrimental effects.

High-load resistance-type exercise is a strong anabolic stimulus that can increase skeletal muscle protein synthesis rates (5, 6), and augment muscle mass and strength when performed as a training program (7). High-load resistance-type exercise training has also been shown effective in counteracting disuse-induced loss of skeletal muscle mass (8). However, performing such demanding exercise might not be feasible for certain (clinical) populations (e.g. rehabilitating athletes, elective surgery patients) who are at risk of losing substantial amounts of muscle mass due to disuse (9).

Recent work suggests that combining low-load resistance-type exercise (LLRE) with blood flow restriction (BFR) represents an effective anabolic stimulus (10-15). Although limited, the available literature suggests that combining LLRE with BFR can increase mixed-muscle protein synthesis rates in healthy young and older participants (12, 13, 15). Furthermore, when applied as a prolonged exercise training intervention, combining LLRE with BFR has also been shown to stimulate skeletal muscle hypertrophy to a similar extent as traditional high-load resistance-type exercise (10, 11, 16, 17).

Interestingly, recent observations in rodents suggest that performing BFR without the addition of LLRE can also induce anabolic effects (18). More specifically, Wistar rats subjected to repeated cycles of BFR under resting conditions showed an acute increase in skeletal muscle p70S6-kinase phosphorylation, a downstream target of the mTOR pathway associated with protein synthesis (18). In line, another study in rodents showed an increase in skeletal muscle fiber size following 6 weeks of repetitive cycles of BFR performed without concomitant exercise training (19). These observations suggest that the application of blood flow restriction under resting conditions stimulates skeletal muscle hypertrophy, but evidence for this in humans is not yet available. In the current study, we assessed the effects of blood flow restriction with and without low-load resistance-type exercise on myofibrillar protein synthesis rates *in vivo* in humans.

Methods

Subjects

Twenty young, healthy male subjects (age: 24±1 y, weight: 72±2 kg, BMI: 22.9±0.6 kg/m^2) participated in this randomized controlled study. The participants were recreationally active and exercised no more than 3 d/wk, with resistance-type exercise being performed no more than 1 d/wk. All subjects were informed about the experimental procedures and possible risks of participation prior to signing an informed consent. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre+, The Netherlands, and was registered at the Nederlandse Trial Register (NTR5914). All procedures were carried out in accordance with the standards stated in the most recent version of the Helsinki Declaration.

Study design

This study was a randomized controlled trial in which subjects were randomly allocated to the group that performed low-load resistance-type exercise (LLRE) or the group that remained in resting conditions (REST). Subsequently, within each group separately, we used a within-subject unilateral-leg design, where one leg was randomly subjected to two 5-min cycles of blood flow restriction, while the contralateral leg served as the within-subject non-BFR control leg. Myofibrillar protein synthesis rates were assessed during a 5-h post-BFR period by combining a primed continuous L-[ring-$^{13}C_6$]phenylalanine infusion with the collection of blood samples from a dorsal hand vein catheter, and muscle biopsies from both the blood flow restricted and control leg in each participant to assess the effect of BFR when combined with LLRE as well as under resting conditions.

Pretesting

Prior to being included in the study, each subject first completed a screening session (≥ 5 d prior to test day) that consisted of assessing health status through a medical questionnaire and measurements of weight and height. Eligible subjects were then randomized to either the LLRE (*n*=10), or REST group (*n*=10). Participants randomized to the LLRE group were familiarized with the leg press and leg extension machines (Technogym, Rotterdam, the Netherlands) and their one-repetition maximum (1RM) was estimated using the multiple repetitions testing procedure (20). The 1RM testing was preceded by a short warm-up set of 15 submaximal repetitions, followed by a maximum of 5 sets of exercise at progressively increasing loads until failure. As a result of the unilateral design of the study (one blood flow restricted leg and the other leg as control), the 1RM of each leg was determined separately. The 1RM was used to calculate the 20%

1RM load required for the leg press and leg extension exercise performed during LLRE on test days, similar to previous studies (12, 13, 16, 21).

Standardization of physical activity and diet

All participants were instructed to refrain from any sort of strenuous physical activity in the 48 h prior to the test day, and to avoid consumption of caffeine and alcohol in the 12 h and 24 h preceding the test day, respectively. They were also instructed to consume a standardized dinner the day before the test. The standardized meal had the same composition for all subjects (62±2 kJ/kg body weight, providing 37 energy% (En%) carbohydrate, 36 En% fat, and 27 En% protein). The standardized dinner was the last meal the subjects consumed before 10:00 p.m. the day before the test day. Thereafter, subjects remained fasted until the end of the test day, but were allowed *ad libitum* consumption of water.

Experimental protocol

Subjects reported to the laboratory by car or public transport at 08:00 a.m. on the test day following an overnight fast. The experimental protocol is depicted in **Figure 6.1**. The test day started by placement of a catheter into an antecubital vein for the stable-isotope amino acid infusion, and a second catheter in a dorsal hand vein of the contralateral arm for arterialized blood draws. To allow sampling of arterialized blood, the hand was first placed in a hot box (60°C) for 10 min before drawing blood.

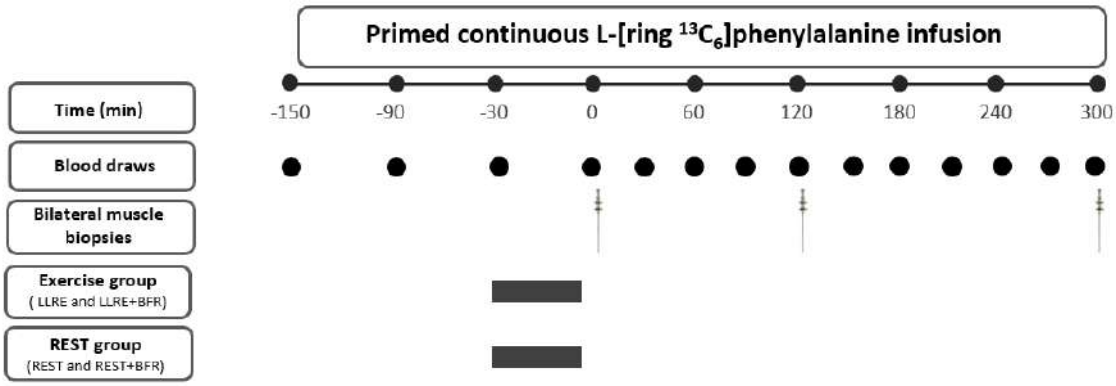

Figure 6.1. Schematic representation of the experimental protocol. The exercise group performed low-load resistance-type exercise (LLRE leg vs LLRE+BFR leg; *n*=10) and the REST group remained in resting conditions (REST leg vs REST+BFR leg; *n*=10)

After collection of a basal blood sample, the plasma phenylalanine pool was primed with a single dose of L-[ring-$^{13}C_6$]phenylalanine (2.25 µmol/kg), after which a continuous L-[ring-$^{13}C_6$]phenylalanine (0.05 µmol/kg/min) intravenous infusion was initiated, lasting

until the end of the test day (*t* = -150 min until *t* = 300 min). Subjects rested in a supine position for another 120 min, while a second and third arterialized blood sample were collected 60 min (*t* = -90 min) and 120 min (*t* = -30 min) into the stable-isotope infusion period, respectively. Subjects then received BFR on one leg for 2x5 min. The BFR approach applied (with respect to cuff size and absolute pressure) resembled that of Gundermann *et al.* who observed a 49% increase in mixed muscle protein synthesis rates following LLRE+BFR (14). We used a 13 cm wide nylon pressure cuff with a 12-cm pneumatic bag inside (Hokanson, SC12; 13×85 cm, Bellevue, WA, USA) that was placed on the proximal part of the thigh and connected to a rapid cuff inflator (ID, Maastricht University Medical Centre+, the Netherlands). The other leg served as the within subject control by not receiving the BFR stimulus. Each of the 2 cycles of BFR was initiated by inflating the cuff to a pressure of 120 mmHg for 30 sec, followed by 10 sec of deflation. This procedure was then repeated 3 more times in total while cuff pressure was increased with 20 mmHg increments (140, 160 and 180 mmHg), before finally reaching the target pressure of 200 mmHg which was maintained for 5 min, as has been done in previous studies (12, 13, 22). Although a reliable measure of the arterial restriction of blood flow during BFR is still lacking (17), we crudely assessed whether cuff inflation resulted in ischemia/hypoxia in the leg during BFR by placing a pulse oximeter on the big toe of each participant and measuring oxygen saturation. Oxygen saturation was within normal range (98-100%) at baseline prior to cuff inflation in all subjects, but reduced to an unmeasurable range when the final pressure of 200 mmHg was reached (i.e., the oximeter ceased measuring shortly after the saturation reduced to 85-90%). Oxygen saturation returned to baseline values 30-60 sec after pressure was released from the cuff.

Participants allocated to the REST treatment were seated in a semi upright position (Fowler's position) during the 2x5 min cycles of BFR, while the LLRE group performed 4 sets of leg press exercise during the first cycle of BFR (1st set 30 repetitions, followed by 3 sets of 15 repetitions) and 3 sets of leg extension exercise during the second cycle of BFR (3 sets of 10 repetitions). Between each set, participants had a 30-sec resting period during the leg press exercise and a 60-sec resting period during the leg extension exercise, while cuff pressure was maintained at 200 mmHg. The two cycles of BFR were interspaced by 5 min in which the cuff was deflated. For the LLRE group, this period was used to perform the same exercise protocol with the control leg (LLRE at 20% 1RM, without BFR). A metronome was used to assure that participants held the correct cadence of 1.5 sec for the concentric and 1.5 sec for the eccentric phase of the exercise. This resulted in the last repetition of the leg press exercise being completed after ~5 min, followed by cuff deflation. For the leg extension exercise, cuff pressure was maintained for another ~1.5 min following the last repetition (at ~3.5 minutes), to complete the 5 min BFR cycle. Both

exercises were performed with a load of 20% 1RM. If a participant failed to complete a set during the 5 min BFR cycle, he would rest until the start of the following set and perform the exercise with a weight that was reduced by 10% of the absolute load.

The last cycle of BFR was followed by a 5-h period in which arterialized blood samples were collected at 30 min intervals (t = 0 - 300 min), and 3 muscle biopsies were collected from both the BFR leg and the control leg (t = 0, 2 and 5 h) to determine myofibrillar protein synthesis rates. Biopsies were collected from the middle region of the *m. vastus lateralis* using the percutaneous needle-biopsy technique under local anesthesia (23). The biopsy samples were collected distal to the area where the BFR cuff was placed, as the area directly underneath the pressure cuff has previously been suggested to show attenuated growth (24). The muscle samples were dissected carefully, freed from any visible adipose tissue and blood, immediately frozen in liquid nitrogen, and stored at -80°C until subsequent analysis. Arterialized blood samples were collected in EDTA-containing tubes and centrifuged at 1000 *g* for 10 min at 4°C. Aliquots of plasma samples were frozen in liquid nitrogen and also stored at -80°C until further analysis.

Plasma and muscle tissue analyses

Plasma phenylalanine concentrations and plasma L-[ring-$^{13}C_6$]phenylalanine enrichments were measured by gas chromatography mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies) as described previously (25). Myofibrillar protein-bound L-[ring-$^{13}C_6$]phenylalanine enrichments were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS; MAT 253, Thermo-Scientific, Bremen, Germany) analysis as described in our previous work (26). Myofibrillar protein-bound enrichments of the t=0 h biopsy was set to 0 for each individual and subtracted from the t=2 and t=5 h enrichments, allowing the t=2 h and t=5 h MPE values to represent the increase compared to the t=0 biopsy.

Western blot analysis was performed on muscle samples homogenized in accordance with previously described procedures (27). The total amount of supernatant that was loaded on gel was based on protein content (50 µg/lane) after a BCA protein assay assessment. With the exception of mammalian target of rapamycin (mTOR) and acetyl-CoA carboxylase (ACC), protein samples were run on a Criterion Precast TGX 4–20% gel (Bio-Rad). The mTOR and ACC proteins were run on a Criterion Precast XT 3–8% Tris-acetate gel (Bio-Rad). Specific proteins were detected with the following antibodies: ACC with anti-ACC and anti-phospho-ACC (Ser^{79}), mTOR with anti-mTOR and anti-phospho-mTOR (Ser^{2448}), S6 protein kinase 1 (p70S6K) with anti-p70S6K and anti-phospho p70S6K (Thr^{389}),

ribosomal protein S6 (RS6) with anti-RS6 and anti-phospho-RS6 (Ser^{235}/Ser^{236}), and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) with anti 4E-BP1 and anti phospho-4E-BP1 ($Thr^{37/46}$) (all from Cell Signaling Technology). Ponceau S staining was used to standardize for the total amount of protein loaded on gel. Additional assessment of total protein within groups and within legs showed no changes over time. Phosphorylation status as a proxy of activation of the signaling proteins was expressed as a ratio, relative to the total amount of each protein.

Determination of skeletal muscle mRNA was performed as previously described (28). Gene Expression Assays (Applied Biosystems) for 18S, mTOR, p70S6K, MuRF1 and MAFbx are listed in **Supplemental Digital Content 6.1**. Statistical analysis for all mRNA data was performed on the delta Ct values. The mRNA data of the control legs (LLRE and REST) at *t* = 0 h were used as reference and were given the value of 1, and all other values from the control and BFR conditions were expressed as fold changes for figure presentation.

Supplemental table 6.1. Gene Expression Assays

Gene Name	Gene Expression Assays
18S	Hs 03003631_g1
MAFbx	Hs 01041408_m1
mTOR	Hs 00234508_m1
MuRF1	Hs 00261590_m1
p70S6K	Hs 00177357_m1

RT-PCR Primers. MAFbx, muscle atrophy F-box; mTOR, mammalian target of rapamycin; MuRF1, muscle RING finger 1; p70S6K, p70 ribosomal protein S6 kinase.

Calculations

In line with previous research (29), myofibrillar protein fractional synthetic rates were calculated using the standard precursor-product equation, as follows:

$$FSR = \frac{\Delta E_{\text{p}}}{E_{\text{precursor}} \cdot t} \cdot 100\%$$

where ΔE_{p} is the increment in myofibrillar protein–bound L-[ring-$^{13}C_6$]phenylalanine enrichment after an incorporation period, $E_{\text{precursor}}$ is the weighted mean plasma L-[ring-$^{13}C_6$]phenylalanine enrichment during that incorporation period, and *t* is the incorporation period (h). Weighted mean plasma enrichments were calculated by taking the average enrichment between all consecutive time points and correcting for the time between

these sampling time points.

Statistical analysis

All data are expressed as means±SEM. A sample size of 10 subjects per group was calculated with a power of 80% and an α-level of 0.05 to detect a 20% difference in myofibrillar protein synthesis rates between the BFR leg and the control leg within groups (effect size: 1.07). All analyses were performed from the period immediately after the BFR intervention, and included the first biopsy (t = 0 min) up until the end of the experimental trial (t = 300 min). Within subject differences in myofibrillar protein synthesis rates between BFR and control leg were assessed using paired samples t-tests, for the resting (REST) and exercise (LLRE) group separately. Although we did not power for between-group differences in myofibrillar protein synthesis rates, we performed unpaired t-tests as secondary analyses on the data to provide insight in potential differences between the REST and LLRE group. Two-factor repeated measures ANOVA was performed with time and treatment (BFR vs control) as within subject factors, to assess differences in ACC, key anabolic signaling proteins of the mTOR pathway and mRNA expression. Observed main effects or interactions were further assessed with Bonferroni-corrected post-hoc testing where appropriate. Mean difference (MD), as well as 95% confidence interval of the difference (95%CI), and Cohen's d_z effect size (calculated as: mean difference / standard deviation of difference) are also presented for the within-subject assessments where appropriate. Statistical significance was set at P<0.05. All calculations were performed using SPSS Statistics (version 25, IBM, Armonk, NY).

Results

All subjects managed to complete the 2x5 min BFR cycles without premature cuff deflation. In the LLRE group, the exercise weight was reduced by 10% during the leg press exercise for two of the 10 subjects, after failing to complete the third set. No adverse events were reported in any of the subjects.

Plasma concentrations and tracer enrichments

Mean plasma phenylalanine concentrations during the experimental trial were 54.3±1.5 µmol/L in the LLRE group, and 55.3±2.7 µmol/L in the REST group, with no differences between groups. Mean plasma enrichments of the infused L-[ring-$^{13}C_6$]phenylalanine during the post-BFR period were 6.88±0.25 and 6.71±0.26 MPE in the LLRE and the REST group, respectively, with no differences between groups. Both phenylalanine concentrations and enrichments were in steady state throughout the experimental period.

Muscle protein-bound enrichments and myofibrillar protein synthesis rates

In the group that performed exercise, post-exercise myofibrillar protein-bound L-[ring-$^{13}C_6$]phenylalanine enrichments at *t* = 2 h did not differ between the LLRE+BFR and the contralateral control leg (LLRE) (0.0064±0.0009 vs 0.0064±0.0011 MPE, respectively; *P*=0.979; MD=0.0000; 95%CI=[-0.0018, 0.0018]; d_z=0.01). At *t* = 5 h, myofibrillar protein-bound enrichments tended to be higher in the LLRE+BFR leg (0.0165±0.0014 MPE), when compared to the LLRE leg (0.0151±0.0014 MPE; *P*=0.051; MD=0.0014; 95%CI=[0.0000, 0.0028]; d_z=0.71). In accordance, myofibrillar protein synthesis rates over 0-2 h did not differ between the LLRE+BFR (0.0463±0.0062 %/h) and LLRE leg (0.0463±0.0078 %/h; *P*=0.997; MD=0.0000; 95%CI=[-0.0128, 0.0128]; d_z=0.001). Also for the 2-5 h period, no significant differences were observed in myofibrillar protein synthesis rates between the LLRE+BFR (0.0486±0.0062 %/h) and LLRE leg (0.0411±0.0037 %/h; *P*=0.186; MD=0.0075; 95%CI=[-0.0043, 0.0193]; d_z=0.45). In contrast, 10±5% higher myofibrillar protein synthesis rates were observed over the entire 0-5 h period in the LLRE+BFR leg when compared to the LLRE leg (*P*=0.042; MD=0.0043; 95%CI=[0.0002, 0.0084]; d_z=0.75; **Figure 6.2**).

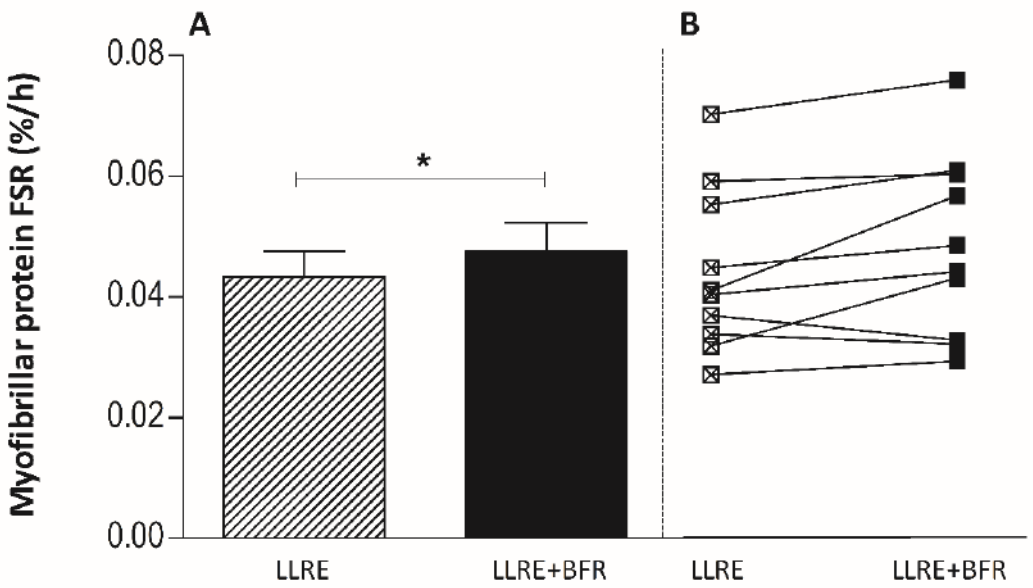

Figure 6.2. Mean±SEM (panel A; *n*=10) and individual (panel B) myofibrillar protein fractional synthetic rates (FSR) measured over a 0-5 h period following low-load resistance-type exercise combined with (LLRE+BFR leg) and without (LLRE leg) blood flow restriction. Data were analyzed using a paired samples t-test (control leg vs BFR leg, within groups). * Indicates a significant difference (*P*<0.05).

In the group that remained in resting condition, myofibrillar protein-bound L-[ring-$^{13}C_6$]phenylalanine enrichments at *t* = 2 h were 0.0056±0.0005 MPE in the REST+BFR leg, and 0.0063±0.0007 MPE in the REST leg, with no differences between legs (*P*=0.344; MD= -0.0007; 95%CI=[-0.0022, 0.0008]; d_z=-0.32). At *t* = 5 h, myofibrillar protein-bound enrichments were increased to 0.0152±0.0013 MPE in the REST+BFR leg, and to

0.0147±0.0012 MPE in the control leg, with no differences between legs (*P*=0.587; MD=0.0006; 95%CI=[-0.0017, 0.0028]; d_z=0.18). In accordance with the myofibrillar protein-bound enrichment data, myofibrillar protein synthesis rates measured over 0-2 h did not differ between the REST+BFR (0.0441±0.0047 %/h) and REST leg (0.0499±0.0064 %/h; *P*=0.296; MD=-0.0058; 95%CI=[-0.0176, 0.0060]; d_z=-0.35). Likewise, no differences were observed in myofibrillar protein synthesis rates over the 2-5 h period between the REST+BFR (0.0447±0.0055 %/h) and REST leg (0.0392±0.0037 %/h; *P*=0.233; MD=0.0055; 95%CI=[-0.0042, 0.0152]; d_z= 0.41). In accordance, myofibrillar protein synthesis rates over the entire 0-5 h period were not different between the REST+BFR and REST leg (*P*=0.683; MD=0.00125; 95%CI=[-0.0055, 0.0080]; d_z=0.13; **Figure 6.3**).

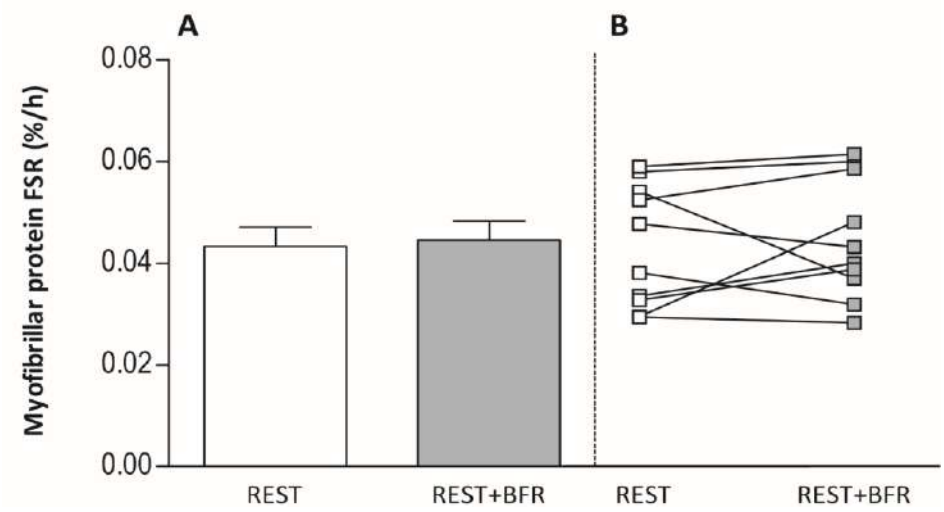

Figure 6.3. Mean±SEM (panel A; *n*=10) and individual (panel B) myofibrillar protein fractional synthetic rates (FSR) measured over a 0-5 h period during resting conditions with (REST+BFR leg) and without (REST leg) blood flow restriction. Data were analyzed with paired samples t-test (control vs BFR leg, within groups). No significant differences were observed between the treatment legs.

In addition to the primary, within-group analyses, the secondary between-group analyses (unpaired t-test rested vs exercised group) showed no differences in myofibrillar protein synthesis rates between REST+BFR and LLRE+BFR, nor between REST and LLRE (*P*≥0.213).

Signaling proteins

In the group that performed exercise, a significant time effect (*P*=0.037) and treatment effect (*P*=0.046) was observed for ACC phosphorylation (**Figure 6.4**), indicating an overall higher ACC phosphorylation in LLRE+BFR vs LLRE, and an overall lower ACC phosphorylation at 2-h vs 0-h (*P*=0.024).

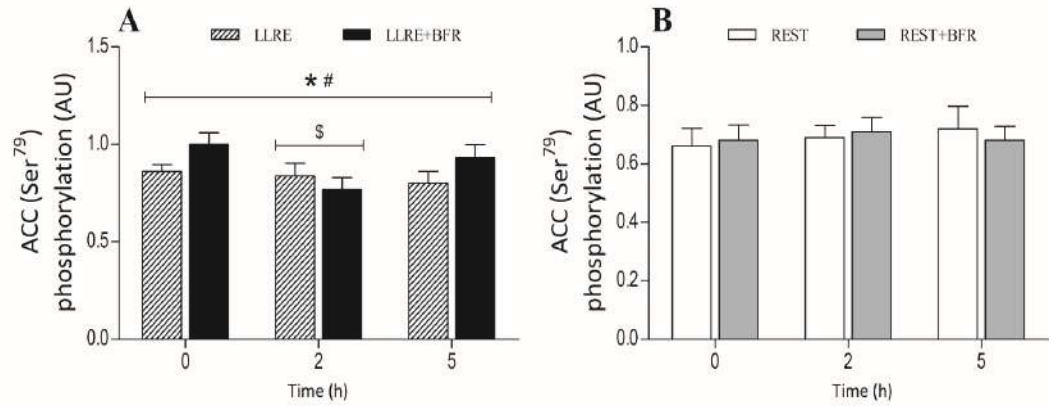

Figure 6.4. Mean±SEM skeletal muscle phosphorylation status of Acetyl-Coa Carboxylase (ACC), following low-load resistance-type exercise (panel A) with (LLRE+BFR; *n*=10) and without (LLRE; *n*=10) blood flow restriction. Mean (±SEM) skeletal muscle phosphorylation status of ACC, during resting conditions (Panel B) with (REST+BFR) and without (REST) blood flow restriction. Data were analyzed with 2-way repeated measures ANOVA (time x treatment leg) within groups. Differences were only observed within the exercise group. Time effect: *P*=0.037, treatment effect: *P*=0.046. [#] Indicates a significant time effect (*P*=0.037): [$]post-hoc testing for the time effect showed lower ACC phosphorylation at 2-h vs the 0-h time point (i.e., for both legs combined; *P*=0.024).

No differences between legs and/or changes over time were observed for mTOR, p70S6K or RS6 (**Figure 6.5A-C**). For 4E-BP1 phosphorylation, a significant time x treatment interaction was observed (*P*=0.009), with higher 4E-BP1 phosphorylation in the LLRE+BFR vs LLRE leg at *t* = 2 h (*P*=0.038; MD=0.081; 95%CI=[0.0058, 0.1564]; d_z=0.77; **Figure 6.5D**).

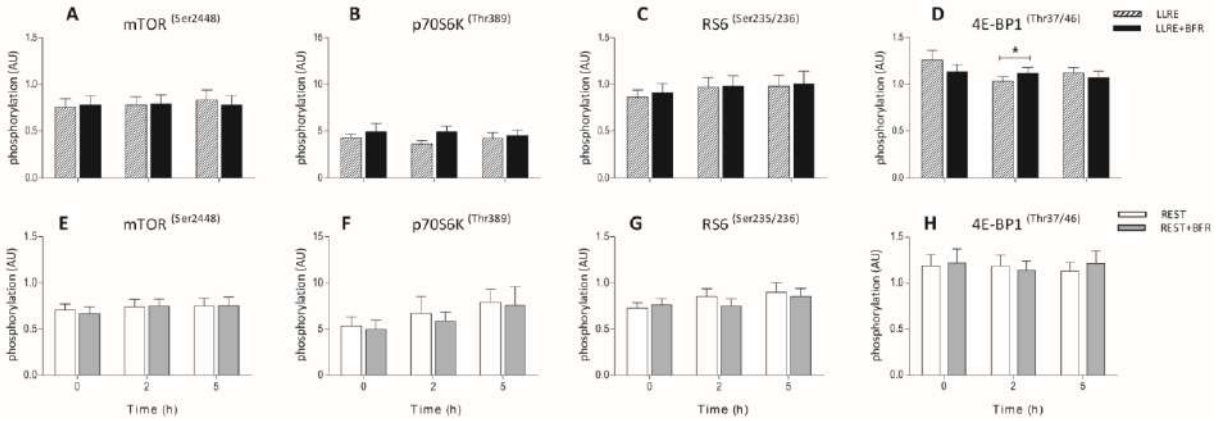

Figure 6.5. Mean±SEM skeletal muscle phosphorylation status of selected anabolic signaling proteins, following low-load resistance-type exercise (panels A-D; *n*=10) and during resting conditions (panels E-H; *n*=10). Data were analyzed with 2-way repeated measures ANOVA (time x treatment leg) within groups. Differences were only observed within the exercise group. 4E-BP1: time x treatment leg interaction: *P*=0.009. * Indicates a significant difference when compared with LLRE at the same time-point (*P*=0.038).

In the group that remained in resting condition, no differences between legs or changes over time were observed for any of the proteins measured (**Figure 6.5E-H**).

mRNA expression

In the group that performed exercise, a time x treatment interaction was observed for MuRF1 (P=0.002), with post-hoc analysis showing higher mRNA expression for LLRE+BFR when compared to LLRE at t = 2 h (P=0.001; MD=0.857; 95%CI=[0.4799, 1.2341]; d_z=1.626; **Figure 6.6A**). For the LLRE+BFR leg, greater MuRF1 mRNA expression was also observed at t = 2 h when compared to the other time points (P<0.01). Furthermore, MuRF1 mRNA expression was lower at t = 5 h when compared to the other time points for both the LLRE+BFR and LLRE leg (P<0.01). No differences between legs or changes over time were observed for MAFbx, mTOR or p70S6K mRNA expression in the LLRE group.

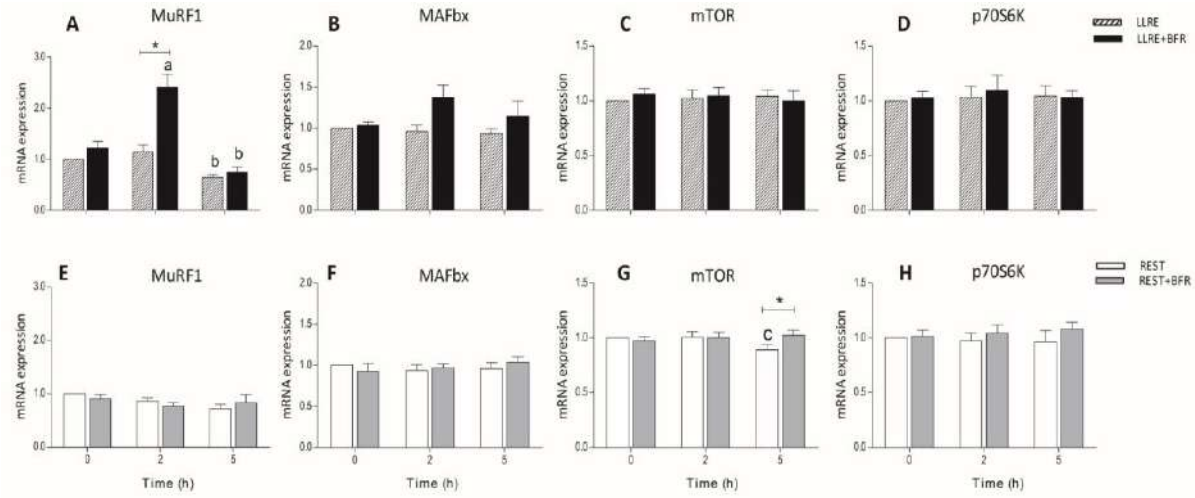

Figure 6.6 Mean±SEM skeletal muscle mRNA expression of selected genes, following low-load resistance-type exercise (panels A-D; n=10) and during resting conditions (panels E-H; n=9). Data were analyzed with 2-way repeated measures ANOVA (time x treatment leg) within groups. Exercise group: MuRF1 time x treatment leg interaction: P=0.002. *Indicates a significant difference (P<0.05). [a] Significantly different from corresponding treatment leg at t = 0 and t = 5 h (P<0.01). [b] Significantly different from corresponding treatment leg at t = 0 and t = 2 h (P<0.01). Resting condition group: mTOR time x treatment leg interaction: P=0.013. [c] Significantly different from corresponding treatment leg at t = 2 h (P=0.027).

In the group that remained in resting condition, mTOR showed a time x treatment interaction (P=0.019), with higher mRNA expression observed for REST+BFR vs REST at t = 5 h (P=0.004; MD=0.147; 95%CI=[0.0610, 0.2330]; d_z =1.22; **Figure 6.6G**). Within the REST leg, mTOR mRNA expression at t = 5 h was observed to be lower than t = 2 h (P=0.027). No further differences were found in mRNA data between the REST+BFR and REST leg (Figure 6.6).

Discussion

The aim of the current study was to assess the effect of blood flow restriction (BFR) with and without low-load resistance-type exercise (LLRE) on myofibrillar protein synthesis rates *in vivo* in healthy young men. Combining LLRE with BFR resulted in higher myofibrillar protein synthesis rates than LLRE alone, whereas BFR applied during resting conditions did not change myofibrillar protein synthesis rates in healthy young men.

Although high-load resistance-type exercise has been shown to be a strong stimulus for skeletal muscle hypertrophy (7), injured athletes or rehabilitating patients might be limited in their ability to perform high-load exercise. An alternative approach suggested to promote skeletal muscle anabolism without the need of heavy weight resistance is the application of BFR with, or even without LLRE (16, 18, 19). As there are only limited data available of the acute anabolic response to BFR under both conditions, the current study assessed the effects of BFR on myofibrillar protein synthesis rates in healthy young men. Using a within-subject unilateral design, the effect of BFR was assessed during low-load resistance-type exercise (LLRE+BFR vs LLRE), as well as during resting conditions (REST+BFR vs REST).

We observed that myofibrillar protein synthesis rates over the 0-5 h period were higher with LLRE+BFR when compared to performing an identical bout of LLRE without BFR (Figure 6.2). These observations are in line with the limited but consistent findings by others (12-15). For example, the first study in this area by Fujita *et. al* (13), showed increased *mixed-muscle* protein synthesis rates measured over 3 h following a single cycle of BFR combined with 20% 1RM LLRE (13). Using a similar BFR protocol (200 mmHg cuff pressure and multiple exercise sets within a BFR cycle, albeit with a wider cuff), the current study confirms and extends on those findings by showing that combining BFR with LLRE increases *myofibrillar* protein synthesis rates. As such, the current study for the first time shows that LLRE+BFR induces an anabolic effect on the contractile protein pool of skeletal muscle tissue.

While the exact mechanism behind the anabolic effect of LLRE+BFR is still unclear, the occurrence of metabolic stress has been proposed to play a role (30). Increased metabolic stress during LLRE+BFR is believed to result in greater skeletal muscle activation by speeding up type I muscle fiber fatigue, and stimulating early recruitment of type II muscle fibers (30, 31). As metabolic stress has been shown to increase AMP activated protein kinase (AMPK) activity (32), the current study included measures of intramuscular ACC phosphorylation, which is a downstream target of AMPK. Phosphorylation of ACC has

been shown to strongly correlate with changes in AMPK activity (33), and was therefore used as a proxy of metabolic stress. In line with several studies that found greater metabolic stress by measuring systemic plasma lactate concentrations (12, 13, 31), we observed higher phosphorylation of ACC following LLRE+BFR when compared to LLRE (Figure 6.4A). Although caution is warranted given the small differences observed, our findings suggest greater intramuscular metabolic stress when combining LLRE with BFR.

Because stimulation of mTOR and its downstream effectors has been shown to correlate with increased muscle protein synthesis rates (34), we also assessed whether phosphorylation of several key anabolic signaling proteins (i.e., mTOR, p70S6K, RS6, and 4E-BP1) differed between LLRE+BFR and LLRE. Performing LLRE+BFR resulted in a small but significantly greater phosphorylation of 4E-BP1 at t =2 h when compared to LLRE (7±9%; Figure 6.5D). Notably though, differences of approximately the same magnitude but in opposite direction were observed for 4E-BP1 phosphorylation at t = 0 h and t = 5 h, although these did not reach statistical significance. Other anabolic signaling proteins also showed no differences between LLRE+BFR and LLRE (Figure 6.5A-C). This general lack of difference may however be related to the timing of biopsies, as previous studies did observe higher p70S6K phosphorylation following a similar LLRE+BFR protocol (12, 13). Thus, although from previous work it appears that the anabolic effect of LLRE+BFR may at least partly be mediated by the mTOR signaling pathway, the current findings do not support a major role.

In view of the fast, albeit transient, increase in gene expression generally observed following exercise, we also determined whether LLRE+BFR vs LLRE showed differences in transcriptional activation during the post-exercise period. While we did not observe changes in mRNA expression of genes associated with muscle protein synthesis (p70S6K and mTOR), LLRE+BFR induced greater expression of the muscle-specific ubiquitin ligase MuRF1 when compared to LLRE (Figure 6.6A). Elevated MuRF1 mRNA expression has frequently been observed early into the recovery period following high-load resistance-type exercise (35, 36), as well as following acute and chronic LLRE+BFR (24). This may be associated with increased protein breakdown as an inherent part of the post-exercise muscle remodeling process (35). In addition, there are some suggestions that MuRF1 is also involved in the regulation of energy metabolism, especially under conditions of metabolic stress (37). The latter would be in line with the greater ACC phosphorylation observed in the present study (Figure 6.4A), and suggests certain homeostatic perturbations with LLRE+BFR, perhaps similar to what has been observed with high-load resistance-type exercise (35, 36). Collectively, the current findings indicate that combining

LLRE with BFR stimulates skeletal muscle remodeling to a greater extent than a comparable bout of LLRE, by increasing anabolic protein signaling as well as promoting protein turnover related gene expression, and, more importantly, by increasing myofibrillar protein synthesis rates.

Based on rodent data suggesting that BFR performed at rest might also stimulate skeletal muscle anabolism (18, 19), we also determined whether BFR could increase myofibrillar protein synthesis rates in the absence of concomitant exercise (REST+BFR). In contrast to our hypothesis, we observed no differences in myofibrillar protein synthesis rates (Figure 6.3), or phosphorylation of anabolic signaling proteins (Figure 6.5) in the REST+BFR leg when compared to REST. We also observed no differences in metabolic stress between REST+BFR and REST as assessed by ACC phosphorylation (Figure 6.4B). Although animal data presented by Nakajima *et al*. also observed no changes in metabolic stress following repeated bouts of REST+BFR vs REST (quantified as AMPK phosphorylation), they did observe greater p70S6K and ribosomal S6 phosphorylation in skeletal muscle tissue (18). It could be argued that between species differences may explain the discrepancy between that study and the current study. Yet, using a longitudinal design in humans, Takarada and colleagues (22) showed that the daily application of BFR without exercise was effective in attenuating skeletal muscle mass loss in subjects undergoing 14 days of non-weight bearing leg immobilization. As the decrease in skeletal muscle mass during disuse has been associated with substantial reductions in muscle protein synthesis rates (38), it could be speculated that the atrophy-attenuating effects of REST+BFR would primarily affect muscle protein synthesis rates. However, the fact that we did not observe an effect of REST+BFR on myofibrillar protein synthesis rates (Figure 6.3) may suggest REST+BFR to only be effective in stimulating skeletal muscle protein synthesis during disuse, rather than further increasing muscle protein synthesis rates in habitually active individuals. Alternatively, it could be speculated that the number of BFR cycles, which were purposely kept similar between REST+BFR and LLRE+BFR in the current study (two cycles of 5 min), may have also played a role. Previous studies assessing the atrophy-attenuating effects of REST+BFR instead performed five repetitive 5-min cycles of REST+BFR within a single session. However, as the assessment of a dose-response relationship between the number of REST+BFR cycles and skeletal muscle anabolism is currently lacking, it is unclear whether performing more than two cycles of REST+BFR might have shown greater effects on anabolic signaling or myofibrillar protein synthesis rates.

Clearly, future research will need to provide further insight as to whether REST+BFR can be effective in preserving basal and/or post-prandial muscle protein synthesis rates and, as such, maintaining skeletal muscle mass during disuse. Furthermore, the contribution of

factors such as the number of repetitive BFR cycles within each session, but also the number of sessions performed daily, as well as the (individualized) pressure required to stimulate skeletal muscle anabolism will need to be further elaborated on to optimize recommendations for BFR performed at rest, as well as when combined with exercise. With respect to individualized pressure, it is important to note that the extent of BFR depends on the specific combination of factors such as cuff width and the pressure applied. How these impact the muscle protein synthetic response to BFR remains to be established, as these factors have until now only been associated with differences in the cardiovascular and perceptual response (39, 40).

Over the past years, a growing number of studies reported increases in muscle mass when BFR is combined with LLRE (10, 11, 16). The present study is the first to show that this is attributed, at least partly, to an increase in *myofibrillar* protein synthesis rates. Applying LLRE+BFR may therefore be useful to maintain skeletal muscle mass and stimulate muscle hypertrophy in a clinical setting, when individuals are unable to perform exercise at a high resistive load (e.g. for injured athletes or as pre- and post-operative training programs).

In conclusion, blood flow restriction performed at rest does not increase myofibrillar protein synthesis rates *in vivo* in humans. When combined with low-load resistance-type exercise blood flow restriction further increases post-exercise myofibrillar protein synthesis rates.

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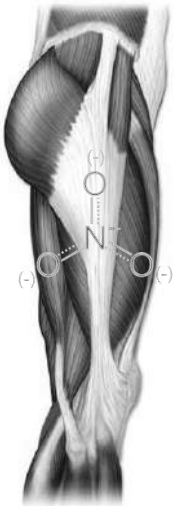
Chapter 7

General discussion



Extensive research performed in the past years has resulted in dietary nitrate currently being considered a nutritional supplement that can improve exercise performance. In this thesis, we assessed several timely questions regarding dietary nitrate supplementation and the associated effects on exercise performance and health, adding to the body of evidence that supports the beneficial effects of dietary nitrate. Notwithstanding such benefits, it has proven difficult to connect the pharmacodynamic effects observed following ingestion of dietary nitrate to *in vivo* kinetics of dietary nitrate. This final chapter will put the findings of this thesis in a broader perspective by discussing the possibility of skeletal muscle tissue serving as an endogenous buffer of nitrate in humans, and the potential consequences this could have. As this is currently an interesting area that will need further exploration, suggestions for future research will also be shortly discussed.

Dietary nitrate metabolism *in vivo* in humans

It is currently accepted that apart from being metabolites of the nitric oxide synthase (NOS) pathway, both nitrate and nitrite can also serve as precursors of nitric oxide (NO) through the nitrate-nitrite-NO pathway. Following digestion and absorption of nitrate-rich food, plasma nitrate concentrations rise, followed by nitrate being actively taken up by the salivary glands and concentrated in saliva (1). Approximately 20% of the nitrate (re-)entering the oral cavity through the salivary glands is reduced to nitrite by facultative bacteria residing on the dorsal part of the tongue (2-4). The nitrite is subsequently swallowed, absorbed into the circulation from the intestines (increasing plasma nitrite concentrations), and transported to other parts within the body where further reduction to NO and other nitrogen oxides is reported to occur through various pathways (5-8). Evidence of this entero-salivary path of nitrate has been provided by prohibiting subjects from swallowing their saliva following the ingestion of dietary nitrate, effectively attenuating the characteristic increase in plasma nitrite concentrations (9). In support of this, we observed substantial increases in both saliva and plasma nitrate and nitrite concentrations ~ 2.5 h following beetroot juice ingestion (**chapter 4**). Although the associated increases in plasma nitrate and nitrite concentrations have since been reported by us in **chapters 2, 3, 5** and (10), as well as by others (11-13), there is still limited insight in the metabolic fate of the ingested nitrate and nitrite *in vivo*. Research performed in animal models in the early 1980's provided some insight using radioactively labeled nitrate and nitrite, showing that within 30 min following administration both anions are swiftly distributed through the systemic circulation to many organs (14). Recent observations in rodents suggest skeletal muscle tissue to possibly serve as an endogenous nitrate buffer (15). In line with these findings, we in **chapter 5** for the first time showed that nitrate is not only present in human plasma but also in skeletal muscle tissue. In fact, we observed

nitrate concentrations in skeletal muscle tissue to be higher than post-absorptive plasma nitrate concentrations, and that ingestion of a nitrate bolus increases nitrate concentration in muscle tissue. These findings suggest that skeletal muscle tissue may be capable of buffering nitrate originating from exogenous (dietary nitrate) and/or endogenous (as a metabolite of the L-arginine and NOS pathway) sources. Whether this is indeed the case is currently unclear, but it is interesting to speculate on whether skeletal muscle tissue may serve as an endogenous nitrate storage site in humans.

Skeletal muscle nitrate content

Our observations of higher nitrate concentrations in skeletal muscle tissue when compared with plasma implies storage of nitrate in skeletal muscle. Such local storage of nitrate could allow faster local NO production through the nitrate-nitrite-NO pathway when NOS-dependent NO production is limited. However, it is not fully clear how or where nitrate might be stored in muscle tissue. As methodological limitations still prevent the exact localization of the cellular distribution of nitrate in skeletal muscle tissue, we can currently only speculate on this matter. As some of the performance enhancing effects associated with nitrate supplementation are believed to result from increased blood flow, it could be hypothesized that a nitrate reservoir might be in close proximity of blood vessels. This is further supported by the fact that some of the enzymes suggested to be capable of reducing (nitrate and) nitrite to NO are present in, or close to blood vessels, including deoxygenated myoglobin and xanthine oxidoreductase (XOR) (5, 16, 17). However, it is questionable whether such co-localization of the nitrate buffer close to blood vessels would allow the generated NO to facilitate certain intramuscular effects that are attributed to nitrate, but that do not necessarily take place in the vicinity of the capillary bed. Effects such as improvements in sarcoplasmic reticulum Ca^{2+} handling (suggested to enhance muscle contractility discussed in **chapter 4**), and a reduction in mitochondrial uncoupling (suggested to account for a reduced oxygen cost discussed in **chapter 2**) have indeed been observed following dietary nitrate ingestion (18, 19). Perhaps that the highly diffusive nature of NO and the intracellular signal transduction of NO through the second messenger cGMP might facilitate these intracellular processes (20). Another explanation could be that nitrate reservoirs may be present in different subcellular locations, as has previously been reported for other muscular substrates. The storage of glycogen is a good example of this as observations in human and rodent skeletal muscle tissue have shown glycogen to be stored in the intramyofibrillar, intermyofibrillar, and subsarcolemmal space (21, 22). Clearly, although recent animal data and our findings in human skeletal muscle tissue support the existence of a skeletal muscle nitrate buffer, there is currently a lack of knowledge on its subcellular localization.

Could nitrate storage in muscle be fiber type specific?

The fact that it is still unclear where nitrate is buffered in skeletal muscle tissue also leaves room for speculation on whether the localization may be associated with the pharmacodynamic effects observed following dietary nitrate ingestion. Accumulating evidence suggests that dietary nitrate might preferentially exert beneficial effects on activities that strongly rely on type II muscle fiber recruitment (23). This is largely based on key studies by Ferguson *et al.* and Hernandez *et al.* with exercising rodents, showing increased blood flow and enhanced muscle contractility primarily in type II skeletal muscle fibers following dietary nitrate supplementation (19, 24). Furthermore nitrite has been observed to show greater reduction to NO in low oxygen conditions (25, 26), underlining why type II muscle fibers and activities that strongly recruit these fibers are believed to benefit from nitrate ingestion (23, 27).

In line with this notion, observations in athletes suggest substantial performance benefits following dietary nitrate ingestion during high-intensity intermittent-type exercise activities, as well as when exercising while under hypoxia. We for example showed that a multiday dietary nitrate ingestion protocol improved repeated high-intensity intermittent-type running performance in trained soccer players (**chapter 4)**, which is supported by observations by others (28-30). Interestingly a study performed by Hoon *et al*. (31) nicely illustrated, using the blood flow restriction procedure, that dietary nitrate ingestion can improve exercise performance during local hypoxia. The authors in that study observed an increased exercise tolerance following dietary nitrate ingestion and suggested this effect to result from greater NO bioavailability, most likely improving performance of type II muscle fibers (23). An interesting question that arises from this explanation is whether the short half-life of NO and nitrite (milliseconds and several minutes, respectively) would require them to be reduced/produced in close proximity of the muscle fibers? Buffering of a stable precursor like nitrate close to or even within the fiber might be a solution, as this may allow confining the effect of the bioactive nitrite and NO to the desired area. In line with the above, it could thus be hypothesized that nitrate is primarily stored within type II skeletal muscle fibers. While this is still speculation, it could support some of the observations described in previous chapters of this thesis. For example in **chapter 6**, we observed a lower nitrate content in skeletal muscle tissue of older healthy males when compared with younger healthy males. We speculated on factors that might explain these differences and discussed whether the lower skeletal muscle type II fiber content that is usually observed in older individuals could be accompanied by lower skeletal muscle nitrate concentrations (32). Extending this hypothetical coupling between skeletal muscle nitrate content and type II fiber

content/activity further may also support the convincing body of evidence showing a lack of effect of nitrate supplementation in highly trained endurance athletes. Multiple studies, including our study described in **chapter 2**, have assessed the ability of dietary nitrate to improve exercise performance in endurance trained athletes. However, in contrast to recreational athletes, no exercise performance improvements have been observed in highly trained endurance athletes following dietary nitrate ingestion (**chapter 2**, (33-35)). It has been proposed that the specific adaptations to endurance training leading to an improved oxidative capacity in such athletes may attenuate the occurrence of low oxygen conditions within the exercising muscle (33). In addition, endurance-trained athletes typically have a muscle fiber composition towards more oxidative, type I muscle fibers, and their exercise pattern relies more heavily on the recruitment of these type I muscle fibers (36), which have been suggested to be less nitrate sensitive (19, 23, 24). We propose that gaining further insight in the potential fiber type specific storage and utilization of nitrate might therefore also improve our understanding of physiological differences between populations that may modify the effectiveness of dietary nitrate supplementation (e.g. age, training status, disease state). As with skeletal muscle glycogen and lipid storage, quantification of fiber-type specific storage of nitrate may be achievable through biochemical and histochemical analytical techniques using both animal and human skeletal muscle tissue (21, 37, 38). Efforts will however be required to set up and optimize such methods, as muscle fiber type specific nitrate and nitrite content analyses have not yet been reported.

Skeletal muscle nitrate loading

Assuming that nitrate is indeed buffered in skeletal muscle tissue, what could this mean for current nitrate supplementation strategies? Although research in the past years has tried to define the most effective supplementation strategy with regard to nitrate source (**chapter 3**) and dose, there is still limited insight in the optimum duration of dietary nitrate supplementation to stimulate ergogenic effects. While many studies have used a multiday supplementation approach, several studies instead assessed the effectiveness of a single bolus ingestion of nitrate on exercise performance (39-43). Interestingly, most of the exercise performance benefits observed following multiday supplementation have also been found following ingestion of a single bolus. Indeed, improvements in parameters such as oxygen efficiency, fatigue resistance, and time trial performance have been observed within 4 hours following ingestion of a single bolus of dietary nitrate (11, 41, 43). It therefore remains unclear what the exact benefit is of a multiday nitrate supplementation regimen. There have been some suggestions that certain ergogenic effects resulting from chronic dietary nitrate supplementation may be due to physiological

adaptations at the myocellular level. For example, reductions in oxygen consumption following 3 days of dietary nitrate ingestion have been associated with changes in mitochondrial function believed to unlikely manifest following acute ingestion (18). However, systemic reductions in oxygen requirement following multiple days of dietary nitrate ingestion have been reported without any apparent changes in mitochondrial function in human skeletal muscle tissue (44). Although this does not exclude the possibility that certain adaptations at the muscle level may specifically result from multiday supplementation, the available body of evidence has been unable to pinpoint the mechanisms that would explain exercise performance improvements specifically induced by chronic dietary nitrate supplementation. Nonetheless, there is a general belief that prolonged ingestion of dietary nitrate might result in greater performance benefits than ingestion of a single bolus. The latter is supported by specific studies that comprehensively compared acute and multiday supplementation (43, 45), and was also concluded in a recent meta-analysis (46). As an alternative to inducing structural adaptations at the muscle level, the goal of a multiday nitrate supplementation regimen could be to allow a more sustained increase in plasma nitrate and nitrite concentrations. Based on our data in **chapter 2** and findings by others (34), plasma nitrate concentrations are indeed higher following a multiday nitrate ingestion regimen when compared with a single bolus. Interestingly, recent observations in rodents suggest that daily ingestion of dietary nitrate can also gradually increase nitrate concentrations in skeletal muscle tissue (47). The researchers in that study observed that skeletal muscle nitrate content was very susceptive to changes in habitual dietary nitrate intake, with a low dietary nitrate diet showing a depletion of skeletal muscle nitrate content when compared with a standard nitrate containing diet. In contrast, high dietary nitrate supplemented conditions showed increased skeletal muscle nitrate concentrations compared to a standard diet. In fact, a 'nitrate depletion' (through a seven day low-nitrate diet) and subsequent 'nitrate loading' strategy (seven days of a high-nitrate diet) resulted in significantly higher nitrate concentrations in skeletal muscle tissue than when the high nitrate diet was not preceded by a low nitrate period (47). This suggests that skeletal muscle nitrate storage may show signs of super-compensation following depletion, perhaps similar to what has been reported for glycogen storage (48, 49). Furthermore, with respect to the debate regarding acute or multiday nitrate supplementation strategies, skeletal muscle nitrate content in the rodents was higher after seven days of dietary nitrate ingestion compared with the content observed following acute nitrate ingestion. Thus, although both acute and multiday dietary nitrate supplementation strategies strongly affect skeletal muscle nitrate contents, chronic ingestion may result in a more sustained increase in intramuscular nitrate concentrations. We have illustrated this concept in **Figure 7.1**.

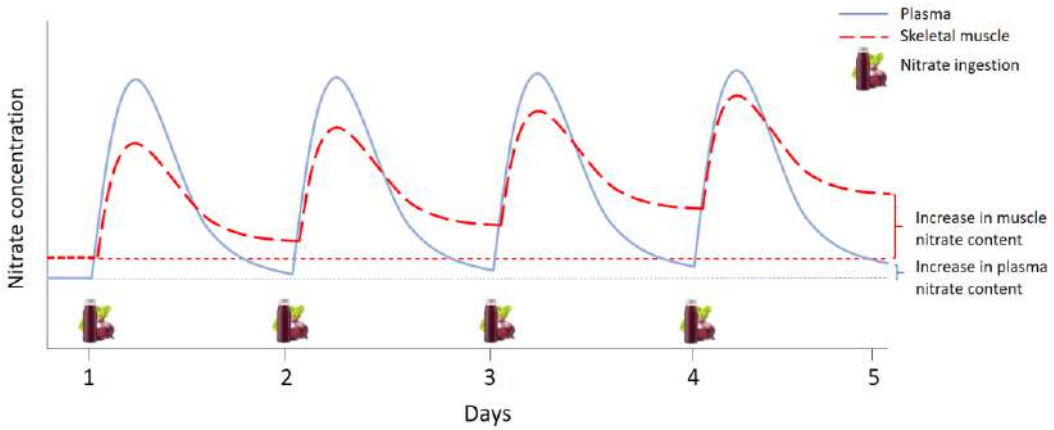


Figure 7.1. Conceptual representation of changes in plasma and skeletal muscle nitrate content following acute and multiday dietary nitrate supplementation

Given the limited data available though, it is currently unknown to what extent various factors such as storage capacity, half-life of nitrate stored in skeletal muscle tissue, as well as the amount and source of dietary nitrate provided may impact the effectiveness of acute versus multiday nitrate ingestion on skeletal muscle nitrate contents. Future studies should provide further insight in these issues by comprehensively comparing the impact of different nitrate supplementation regimens on muscle tissue nitrate content. Observed changes in plasma and skeletal muscle nitrate concentrations may give some indication of the added benefit of a multiday supplementation protocol, especially if these changes can be associated with enhanced exercise performance effects.

What about skeletal muscle nitrite?

Although we previously managed to measure nitrate concentrations in human skeletal muscle tissue in **chapter 5**, we did not detect any nitrite in skeletal muscle tissue in the post absorptive state, as well as following a single bolus ingestion of dietary nitrate. This is most likely due to the fact that the post absorptive and post-ingestion nitrite concentrations present in skeletal muscle tissue remained well below our detection limit of 2 nmol/g·ww^{-1} tissue. Recent observations in rodents suggest basal skeletal muscle nitrite concentrations to be in the range of 0.6 nmol/g tissue, increasing up to ~4-5 nmol/g tissue following three days of dietary nitrate supplementation (47). The daily amount of nitrate per kg bodyweight ingested by the rodents was however ~6 times the acute bolus ingested by our human participants and might therefore have allowed muscle nitrite concentrations to show greater increases. Of note, similar to our study described in **chapter 5**, the concentrations of nitrate and nitrite measured in rodent muscle were assessed under resting conditions (47). Perhaps that skeletal muscle nitrite concentrations would have been higher in both studies if exercise or hypoxia was included to stimulate

nitrate to nitrite reduction. Especially (local) hypoxia has been shown to promote nitrate to nitrite reduction in skeletal muscle tissue and in different organs (liver, colon, heart), most likely mediated by the nitrate-reducing enzyme XOR (15, 50-52). Because close associations between changes in nitrite concentrations and several NO-like effects have been observed (i.e., changes in blood pressure, increased blood flow), it would be interesting to determine whether nitrate ingestion combined with exercise and/or hypoxia would show great(er) changes in skeletal muscle nitrite concentrations, thereby exceeding our detection limit of 2 nmol/g·ww^{-1}. A recent study in rodents for example showed a decrease in nitrate and a transient increase in nitrite concentrations in exercised skeletal muscle tissue without prior ingestion of dietary nitrate (53). The authors proposed the increase in nitrite to result from nitrate to nitrite reduction within the skeletal muscle tissue, which was most likely stimulated by the hypoxic and metabolically acidic environment created by the exercise stimulus. Based on these findings, it could be suggested that nitrite concentrations can indeed increase in skeletal muscle tissue, but that a relatively high nitrate dose or, perhaps even more effective, an intervention that stimulates nitrate reduction such as hypoxia or exercise may be required to show detectable nitrite levels in skeletal muscle tissue in humans.

Apart from nitrite being generated from nitrate reduction in skeletal muscle, it is perhaps possible that nitrite itself is also buffered in skeletal muscle tissue. Local storage of nitrite would minimize the reduction steps required to increase NO bioavailability. As we were unable to measure nitrite in human skeletal muscle tissue, we currently cannot confirm nor deny the existence of such a buffer. However, the short half-life of nitrite in blood (~110 s), as well as the reported bioactivity of nitrite as a signaling molecule (54), might arguably favor nitrate to serve as a more stable precursor pool for the nitrate-nitrite-NO pathway in skeletal muscle tissue. As already discussed, this would only seem useful if nitrate can be locally reduced, or transported towards a nearby site where nitrate reduction can take place. While local reduction of nitrate has indeed been shown to occur in skeletal muscle tissue of rodents, there is no data available to support such reduction processes taking place in human skeletal muscle. As such, future work should further establish whether human skeletal muscle tissue is capable of not only buffering, but also reducing nitrate to nitrite locally. Similar to what has been done in rodents, this could be assessed by collecting skeletal muscle samples prior to and following an intervention that promotes nitrate to nitrite reduction; i.e., through exercise and/or hypoxia. One option would be to use the blood flow restriction procedure we used in **chapter 6** to create local hypoxia in the muscle(s) under investigation. This could be further complemented by *in vitro* measurements of nitrate and nitrite reduction activity of human skeletal muscle tissue (53). The *in vivo* measurements may however prove challenging when trying to

distinguish nitrite formed from nitrate reduction and nitrite formed through the L-arginine and NOS pathway. The use of ^{15}N labeled nitrate may provide a solution, as this has previously been applied to track the metabolism of exogenous nitrate (55, 56). This approach may allow further quantification of the *in vivo* reduction of nitrate to nitrite and NO.

Conclusions and suggestions for future research

There is currently limited insight in the exact mode of action underlying the ergogenic effects of dietary nitrate supplementation. There is also very little known about the metabolism of dietary nitrate following ingestion and subsequent appearance of nitrate and nitrite in the circulation. Skeletal muscle is now being proposed as a possible site of nitrate buffering and the data in this thesis provides evidence of the possible existence of such a nitrate buffer in humans. The capacity to store nitrate in skeletal muscle tissue and/or the ability to increase such storage may soon be considered an important factor modulating the effectiveness of dietary nitrate as an exercise performance enhancing aid. However, the exact (intramuscular) location of this nitrate reservoir is still unclear and we can currently only speculate it to be in close proximity of blood vessels and type II skeletal muscle fibers. The latter would be in line with the current notion of a possible fiber-type specific effect of dietary nitrate supplementation. The relevance of such a buffer does however seem to strongly depend on the ability of the stored nitrate to be locally reduced to nitrite. Although evidence of nitrate reduction has been observed in rodent muscle, it is currently unknown whether human skeletal muscle tissue is also capable of doing the same. Future research should therefore elaborate on the following questions:

- Where is the nitrate stored in skeletal muscle tissue located (vasculature, or specific/ multiple subcellular spaces)?
- How is nitrate present in skeletal muscle tissue reduced locally to nitrite?
- Which factors stimulate the utilization of nitrate stored in skeletal muscle tissue?
- How can intake of dietary nitrate be used to affect local storage of nitrate in human muscle?
- How long following a high nitrate diet does nitrate remain stored in skeletal muscle tissue?
- To what extent do factors such as age, disease, type of sport, and training status result in changes in skeletal muscle nitrate content and/or metabolism?

Confirming the existence of the proposed nitrate reservoir as well as the ability of stored nitrate to serve as a nitrite and NO-precursor could open up new avenues for further research. This could include optimization of dietary nitrate supplementation strategies

(regarding source, dose, and duration) based on skeletal muscle nitrate content, or even relating pharmacodynamic effects to changes in skeletal muscle nitrate content and subsequent utilization. The observation of nitrate storage in skeletal muscle tissue described in this thesis could serve as a starting point for future studies to look further into several beneficial health and exercise performance effects attributed to dietary nitrate supplementation. Although we limited our assessment to skeletal muscle tissue, it could be argued that if nitrate can indeed serve as a stable NO-precursor storage pool, reservoirs of nitrate might also be present in other organs for the same reason. Only through elucidation of these gaps in knowledge will we eventually know whether the storage of nitrate in skeletal muscle tissue is as relevant as we imagine it to be.

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Summary

Dietary nitrate is a nutritional supplement that has received a lot of attention in recent years following groundbreaking observations of reduced oxygen cost during submaximal cycling exercise after nitrate ingestion. Many studies have since explored the potential of dietary nitrate to serve as an ergogenic aid, but there is still limited insight in the optimal supplementation strategy. Furthermore, the endogenous conversion of dietary nitrate into nitrite and further into nitric oxide (NO) is believed to be the basic mechanism underlying the beneficial effects of dietary nitrate. However, it is currently still unclear where and/or under what conditions this *nitrate-nitrite-NO pathway* is mostly activated. The studies described in this thesis assessed various factors that may modulate the effects of dietary nitrate supplementation, with the aim to provide further insight into the physiological, pharmacodynamic, and ergogenic properties of dietary nitrate.

Whereas previous work has already established that ~520 mg of dietary nitrate is required to improve exercise performance in recreationally active subjects, one of the remaining questions is whether the duration of dietary nitrate supplementation might influence its effectiveness. In **chapter 2**, we therefore compared the effect of acute versus 6-day dietary nitrate ingestion on submaximal oxygen consumption and 10-km time trial performance in highly trained endurance athletes. We assessed this in trained endurance athletes as the majority of (acute) nitrate supplementation protocols had failed to elicit performance benefits in these athletes. The cross-over design allowed us to determine whether extension of an already high dietary nitrate supplementation strategy (800 mg) from 1 day to 6 days would result in (greater) performance improvements when compared to a placebo. Despite significant increases in plasma nitrate and nitrite concentrations, no reduction was observed in the oxygen cost of submaximal cycling exercise in the trained athletes following either an acute or a 6-day dietary nitrate supplementation protocol. Furthermore, no improvements were observed in 10 km time-trial performance with both supplementation protocols. Based on these findings and similar observations by others, it became apparent that dietary nitrate is probably less effective in improving exercise performance in highly trained endurance athletes.

In **chapter 3**, we assessed the effect of ingesting 800 mg of dietary nitrate in the form of concentrated beetroot juice, sodium nitrate, a rocket salad beverage, or a spinach beverage. We determined whether the ingestion of these sources resulted in different changes in plasma nitrate and nitrite concentrations, and whether the hemodynamic effects differed between sources. We observed similar (~9 fold) increases in plasma nitrate concentrations between the four sources, as well as a similar decrease in diastolic blood pressure (2-8 mmHg) following ingestion of each source. In contrast, a more

pronounced increase in plasma nitrite concentrations was observed following the spinach and rocket salad beverages when compared with the nitrate salt. Most importantly, a decrease in systolic blood pressure was only observed following ingestion of the three vegetable-based sources. We therefore conclude that the ingestion of vegetable-based dietary nitrate sources may be preferred to elicit both pharmacokinetic and pharmacodynamic effects.

In view of the lack of effects in trained endurance athletes as well as the potential preference for vegetable-based nitrate sources, we used beetroot juice in the study described in **chapter 4** to determine whether ingestion of dietary nitrate for multiple days could improve high-intensity intermittent-type exercise performance. Using the Yo-Yo intermittent recovery test, we showed that 6 days of concentrated beetroot juice ingestion increases high-intensity intermittent-type running performance in trained soccer players. This finding is completely in line with the emerging belief that dietary nitrate might primarily exert beneficial effects on type II muscle fibers, which are heavily recruited during high-intensity type exercise.

As the exact mechanism behind the ergogenic effects of dietary nitrate is still unknown, there is ample room to speculate on factors that may be related to the observed effects. For example, observations in animals suggest that nitrate may be stored and locally utilized in skeletal muscle tissue. However, insight in the pharmacokinetics of dietary nitrate in humans has been limited to measuring concentrations in plasma and saliva. In **chapter 5**, we therefore determined the basal nitrate content in human skeletal muscle tissue and compared that to what was present in plasma. We showed that in the basal state, nitrate concentrations are substantially greater in skeletal muscle tissue when compared to plasma concentrations, suggesting a nitrate buffering function of skeletal muscle. In addition, ingestion of a single bolus of dietary nitrate increased both plasma and skeletal muscle nitrate concentrations in the hours following ingestion. The nitrate content of skeletal muscle tissue therefore seems susceptible to dietary nitrate ingestion, and this storage may allow nitrate to serve as a local NO precursor.

A growing number of human and animal studies indicate that dietary nitrate may be most effective during low oxygen and acidic conditions. **In chapter 6**, we assessed the effect of blood flow restriction combined with and without low-load resistance-type exercise on myofibrillar protein synthesis rates. We observed that myofibrillar protein synthesis rates and anabolic signaling increased with blood flow restriction, but only when combined with low-load resistance type exercise. These findings suggest that a transient decrease in

blood flow and oxygen supply may increase the anabolic effect of an otherwise ineffective exercise stimulus. Although we have not yet assessed this, the next step would be to determine whether the nitrate-nitrite-nitric oxide pathway might play a role in inducing this anabolic effect.

In the last chapter, we focus on discussing the implications of nitrate being stored in human skeletal muscle tissue. We address how the different factors assessed in the current thesis (i.e. duration of supplementation, the nitrate source, the type of exercise) as well as other factors described in literature (such as dose, muscle fiber type composition, training status, age) could be associated with the skeletal muscle nitrate content. Based on this thesis, we suggest that the ability to increase both skeletal muscle nitrate content and its local utilization likely represent important factors modulating the effectiveness of dietary nitrate supplementation when aiming to improve exercise performance. So far, it appears that most benefits are expected with high-intensity type exercise rather than endurance type activities, and with vegetable-based nitrate sources rather than nitrate salts. Yet, future work will need to further unravel how intramuscular nitrate storage is affected by the nitrate source, the dose, and the duration of supplementation, as well as by the type of exercise performed.

Samenvatting

In de afgelopen jaren zijn steeds meer sporters het voedingssupplement nitraat gaan gebruiken omdat het prestatiebevorderend zou kunnen werken. Dit gebeurde nadat ongeveer 10 jaar geleden verschillende onderzoeken lieten zien dat de inname van een nitraatsupplement de hoeveelheid zuurstof die nodig is tijdens inspanning kan verlagen. Onderzoek liet ook zien dat nitraatinname de prestatie tijdens een tijdrit op de fiets kan verbeteren. Het doel van de onderzoeken die beschreven staan in dit proefschrift was om verder inzicht te krijgen in het gebruik van nitraatsupplementen om sportprestaties te verbeteren. De bevindingen uit dit proefschrift zijn hier kort samengevat.

Hoewel er nog veel onbekend is over hoe nitraatsupplementen de prestatie kunnen verbeteren, is het algemeen geaccepteerd dat stikstofmonoxide (afgekort als 'NO') hier een belangrijke rol in speelt. Nitraat wordt namelijk na inname eerst in het lichaam omgezet in nitriet (wat o.a. in de mond gebeurt) en daarna in stikstofmonoxide. Stikstofmonoxide is een stof die betrokken is bij veel fysiologische processen in het menselijk lichaam, waaronder het verwijden van de bloedvaten en het faciliteren van spiercontracties. Omdat nitraat ervoor kan zorgen dat er meer stikstofmonoxide in het lichaam beschikbaar komt, hebben veel studies in de afgelopen jaren onderzocht hoe nitraat het beste gebruikt kan worden om de sportprestatie te verbeteren.

Een van de eerste studies die inzicht gaf in hoe nitraatinname geoptimaliseerd zou kunnen worden, liet zien dat er ongeveer 500 mg nitraat nodig is om de sportprestatie bij recreatieve sporters te verbeteren. In navolging hierop hebben wij in **hoofdstuk 2** onderzocht wat het effect is van een 1- of 6-daagse inname van nitraat op de zuurstof consumptie tijdens inspanning, en op de sportprestatie met behulp van een 10-km tijdrit op de fiets. Dit hebben we onderzocht bij goedgetrainde duuratleten omdat veel studies bij deze atleten geen effect van nitraatinname lieten zien, terwijl dit bij recreatieve sporters wel werd gezien. We wilden onderzoeken of dit wellicht aan de duur van nitraatinname lag. Hoewel we in onze studie een duidelijke stijging in bloedconcentraties van nitraat en nitriet zagen door inname van nitraat, bleek dit geen effect te hebben op de zuurstof consumptie tijdens inspanning en ook niet op de prestatie tijdens de 10-km tijdrit. Tezamen met soortgelijke bevindingen door andere onderzoekers concluderen we dat nitraatinname geen effect heeft op de sportprestatie van goed getrainde *duur*atleten.

Naast eventuele verschillen door de duur van nitraatinname (1 of 6 dagen in hoofdstuk 2) wilden we ook graag weten of de keuze van de nitraatbron van belang kon zijn voor de effectiviteit. Om die reden hebben we in **hoofdstuk 3** vastgesteld wat het effect is van nitraatinname in de vorm van rode bietensap, nitraatzout, een spinazie drankje en een

rucola drankje. Naast het effect op de concentraties van nitraat en nitriet in het bloed, hebben we ook gekeken naar het effect op de bloeddruk. Dit onderzoek liet zien dat bij een vergelijkbare hoeveelheid nitraat de inname van verschillende nitraatbronnen resulteert in een vergelijkbare toename van nitraat concentraties in het bloed. De meest opmerkelijke bevinding was echter dat de bloeddruk (bovendruk) alleen daalde na inname van de nitraatrijke groente bronnen, en dus niet na inname van het nitraatzout. We concluderen op basis van deze resultaten dat de inname van plantaardige nitraatbronnen waarschijnlijk effectiever zal zijn dan wanneer een nitraatzout gebruikt wordt.

Met deze kennis hebben we in **hoofdstuk 4** vastgesteld wat het effect is van rode bietensap op de sportprestatie van goedgetrainde voetballers. We onderzochten dit met behulp van de Yo-Yo test, wat een aangepaste 'shuttle-run test' is (ook wel bekend als de 'piepjes test'). Dit onderzoek speelde in op het feit dat in toenemende mate wordt gedacht dat nitraat vooral effect heeft op (kortdurende) explosieve inspanning van zeer hoge intensiteit, in plaats van laag intensiteit *duur*inspanning. Ons onderzoek liet inderdaad zien dat 6 dagen rode bietensap inname leidt tot een verbetering van de sprintcapaciteit bij goedgetrainde voetballers zoals gemeten met de Yo-Yo test. Deze bevinding suggereert dat nitraat een effect kan hebben op de sportprestatie wanneer de spieren een lage zuurstof beschikbaarheid hebben, zoals bij hoge intensiteit en explosieve inspanning. De mogelijke relatie die dit effect zou kunnen hebben met de zuurstofconcentratie in de spier is interessant, omdat onderzoeken in muizen en ratten eerder hadden aangetoond dat nitraat ook in de spier opgeslagen kan worden. Bij mensen werd alleen naar de concentraties nitraat in het bloed gekeken. De onderzoeken in muizen en ratten concludeerden echter dat nitraat wellicht in de spier wordt opgeslagen om stikstofmonoxide te produceren op momenten dat de zuurstofconcentratie laag is. Omdat vergelijkbare gegevens bij mensen nog niet beschikbaar waren besloten wij om dit in **hoofdstuk 5** te onderzoeken. In lijn met de bevindingen in muizen en ratten, zagen we bij mensen dat de spier hogere concentraties nitraat bevat dan de nitraat concentratie in het bloed. Bovendien bleek dat de inname van nitraat ertoe leidt dat niet alleen de nitraat concentratie in het bloed, maar ook de concentratie in de spier toeneemt. Hoewel het nog onduidelijk is of de omzetting van nitraat naar stikstofmonoxide lokaal in de spier mogelijk is en hoe dit dan precies zou werken, is het wel denkbaar dat dit bij kan dragen aan alle gunstige effecten die met nitraatinname worden verkregen.

In het laatste onderzoek, dat beschreven staat in **hoofdstuk 6**, hebben we gemeten wat de kortdurende restrictie van bloed naar de spier doet met de aanmaak van nieuw spierweefsel. Eerder onderzoek had al aangetoond dat het kortdurend verlagen van de

doorbloeding (en daarmee de beschikbaarheid van zuurstof) een gunstig effect kan hebben op de groei en behoud van spiermassa. Omdat voor de omzetting van nitraat naar stikstofmonoxide een omgeving met weinig zuurstof binnen de spier bevorderlijk lijkt, wilden we vaststellen of dit wellicht een verband had met de gunstige effecten op spiermassa. We wilden echter eerst bevestigen dat een kortdurende restrictie van bloed naar de spier bij gezonde jonge mannen voor een toename in spieraanmaak zorgt. Dit hebben we onderzocht door één groep mannen de restrictie van bloedtoevoer met inspanning te laten combineren, terwijl een andere groep geen inspanning uitvoerde en alleen restrictie van de bloedtoevoer als interventie kreeg. Ons onderzoek liet zien dat de aanmaak van meer spierweefsel alleen gestimuleerd wordt wanneer de kortdurende restrictie van bloedtoevoer gecombineerd wordt met inspanning. Of dit ook gekoppeld is aan de omzetting van nitraat naar stikstofmonoxide in de spier is nu nog de vraag, maar op basis van de beschikbare resultaten zou een verband zeker mogelijk zijn. Toekomstige studies zullen hier meer inzicht in moeten geven.

In het laatste hoofdstuk bediscussiëren we tot slot hoe de opslag van nitraat in spierweefsel invloed kan hebben op de effecten die gezien worden op de sportprestatie. Daarbij wordt een aantal factoren besproken die aan bod zijn gekomen in dit proefschrift, zoals de wijze van nitraatsuppletie (de duur van nitraatsuppletie, de nitraat bron, de dosis), maar ook factoren die mogelijk gerelateerd zijn aan de hoeveelheid nitraat in de spier (zoals bijvoorbeeld leeftijd, de mate van getraindheid en mogelijk spiervezel-specifieke effecten). Op basis van de onderzoeken beschreven in dit proefschrift suggereren wij dat de toename in de hoeveelheid nitraat in spierweefsel, en de mate waarin dit nitraat verbruikt wordt tijdens inspanning in belangrijke mate bepalen wat de effectiviteit is van nitraatsuppletie om sportprestaties te verbeteren. Op dit moment is de verwachting dat de grootste effecten te zien zijn tijdens inspanningen met hoge intensiteit, en bij inname van nitraatrijke groenten in vergelijking met nitraat zouten. Toekomstig onderzoek zal echter verder inzicht moeten geven in de mate waarin nitraat concentraties in spierweefsel beïnvloed worden door factoren zoals de nitraat bron, de hoeveelheid nitraat en de duur van nitraatinname, alsmede hoe het type inspanning van invloed is op het verbruik van het nitraat dat in de spier is opgeslagen.

Valorization

Scientific and societal relevance

In the sports nutrition field, meticulous research in the past decades has provided clear insight in the general areas of application for the macronutrients (i.e., carbohydrates as a main fuel, and proteins to support skeletal muscle structural adaptations) resulting in detailed recommendations for specific sports. In contrast, the efficacy of several nutritional supplements to be used as ergogenic aids or to support exercise adaptation is less well established. Dietary nitrate is an example of such a nutritional supplement that gained a lot of attention in recent years as a promising agent to enhance sports performance in athletes of both endurance and high-intensity type sports, and was even named the 'next magic bullet'. However, recommendations on the effective application of a nutritional supplement such as dietary nitrate requires extensive research that provides insight in the various factors that may modulate its efficacy. The studies described in this thesis aimed to do so by determining whether the beneficial effects of dietary nitrate can be associated with factors such as the duration of supplementation, the nitrate source, and the type of sport, while also trying to gain further insight in the *in vivo* pharmacokinetics.

The first study described in this thesis showed that even a multiday supplementation protocol with dietary nitrate does not improve performance in highly trained endurance athletes. For sport supplements in general, it is extremely relevant to not only know who might benefit, but also provide clear recommendations for those that may not benefit from it. Indeed, our findings strongly supported the ongoing paradigm shift, suggesting dietary nitrate to be less effective for endurance athletes, and perhaps more beneficial for exercise intensities that strongly recruit type II muscle fibers. We thereafter performed a study that showed that vegetable-based nitrate sources may induce greater benefits than dietary nitrate salts provided as an extracted powder. The relevance of this finding lies in the fact that although nitrate salts may be considered an easier nitrate source to process industrially into nutritional products (such as sport drinks and dietary powders), they may not be as effective as vegetable-based sources, potentially limiting their suitability as a sport supplement. The knowledge gained from these first two studies was subsequently incorporated into the third study, in which we showed that a multiday nitrate-rich beetroot juice supplementation protocol improves performance during high-intensity intermittent-type exercise in trained soccer players.

The findings from these first three studies have already been disseminated in different ways to educate and advice. For example, the knowledge has been incorporated in several sports and nutrition courses of bachelor and master students at the Maastricht University,

education of sports dietitians at the HAN University of Applied Sciences, and a course on sports nutrition for sports physicians. Furthermore, apart from the scientific publications and scientific conferences in which the observations described in the current thesis have been shared, the findings have also been spread through publications in sports and nutrition based magazines read by the general public. This approach allowed us to provide well-balanced evidence to the general public regarding the potential of dietary nitrate to promote health and exercise performance (i.e., taking our own findings as well as other recent insights into account). To specifically inform athletes and the associated (medical) staff of these athletes, we recently also started updating the dietary nitrate factsheet in collaboration with the Dutch Olympic Committee (NOC*NSF). As factsheets are considered the most comprehensive source of information for competitive athletes, providing state-of-the-art and very applicable insights regarding ergogenic aids, this was and is one of the primary aims of our pre-determined valorization plan.

Innovation

In addition to the more applied findings described in this thesis, we also provided further insight in the metabolic fate of dietary nitrate. We provide the first human evidence that nitrate is stored in skeletal muscle tissue and that this 'reservoir' can be increased with dietary nitrate supplementation. This may be an important step towards understanding how dietary nitrate can improve skeletal muscle function. In fact, we propose that the performance enhancing effect of dietary nitrate may be related to the local storage and subsequent utilization of nitrate. If this hypothesis is true, optimizing dietary nitrate supplementation for exercise performance should be aimed at establishing the dose, source and duration that most effectively increases the nitrate stored in skeletal muscle tissue. Additionally, the ability to locally utilize the nitrate stored in muscle may represent a crucial factor explaining inter-individual differences in the responsiveness to dietary nitrate. Although further work is needed to confirm these concepts, our findings clearly call for explorative research approaches with the aim to improve the local utilization of the stored nitrate, consequently increasing the effectiveness of a supplementation protocol. Furthermore, although we did not specifically focus on the clinical application of dietary nitrate, it could be speculated that the local storage of dietary nitrate may also have a functional purpose in other organs. Several organs have indeed been suggested to be capable of locally reducing nitrate and nitrite into nitric oxide, which may allow for vasodilation during ischemic and/or hypoxic events. Reducing the negative impact of such events could be crucial in maintaining the function of several organs. The fact that there is greater utilization of nitrate during low oxygen conditions further supports the potentially major role that nitrate may have in such situations, and underlines the need

to further establish the local storage and utilization of dietary nitrate beyond that in skeletal muscle tissue.

Concluding remarks

The studies described in this thesis have allowed for a further optimization of dietary nitrate supplementation strategies when aiming to improve exercise performance. Furthermore, the observed local storage of nitrate in skeletal muscle tissue provides new insights in the metabolic fate of dietary nitrate *in vivo*, and provides opportunities for future research to unravel the mechanisms behind the ergogenic effect of dietary nitrate. In addition to the relevance this may have for supplementation strategies to improve exercise performance, the observed local storage of nitrate also reveals a knowledge gap in an area with perhaps even greater implications. Extending the benefit of local storage and utilization of nitrate in skeletal muscle tissue to other organs (such as the brain and heart) provides possibilities for nitrate supplementation to attenuate tissue damage and dysfunction during an ischemic and/or hypoxic event. Confirming this hypothesis in future research will open up new avenues for the clinical application of dietary nitrate.

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Curriculum Vitae

Jean Nyakayiru was born on July 23, 1987 in Nairobi, Kenya. At the age of six, Jean moved to the Netherlands together with his family and has lived there ever since. He completed secondary school at the Elzendaal College in Boxmeer in 2005. He then continued to study physical therapy at the HAN University of Applied Sciences and obtained his bachelor in 2009. In that same year, he started a pre-master, followed by the full master in Clinical Human Movement Sciences at the Radboud University, which he completed in 2012. During the last year of his master, he performed a 7-month internship at the Liverpool John Moores University in the United Kingdome under supervision of Prof. dr. Dick Thijssen and Prof. dr. Helen Jones. During that internship, he conducted a study assessing the effect of 8-weeks of repeated ischemic preconditioning on arterial and cutaneous microcirculatory function in healthy young men.

After obtaining his MSc in 2012, he stared working as a research assistant at the department of Integrative Physiology at Radboud University in the Netherlands, in the group of Prof. dr. Maria Hopman and Prof. dr. Dick Thijssen. He worked on several projects during that period, including the 4-day Marches project, and a study assessing the effect of flavonoid-rich black tea and red beetroot juice on the glucose homeostasis and peripheral vascular resistance in insulin-resistant obese men. Following that year as a research assistant, Jean started a PhD project under supervision of dr. Lex Verdijk and Prof. dr. Luc van Loon at the Maastricht University in the Netherlands. During his PhD, Jean performed multiple human *in vivo* studies assessing the ergogenic potential of dietary nitrate. Throughout the years, Jean has published several papers from his PhD project and has presented his work at international conferences. During the European College of Sport Science conference in 2017, Jean was awarded the Young Investigators Award for his mini-oral presentation on the nitrate content of human skeletal muscle tissue. A year later, Jean was awarded a Young Investigators Award from the European College of Sport Science for his oral presentation on the effect of blood flow restriction with and without low-load resistance type exercise on myofibrillar protein synthesis rates.

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