From the Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

DIETARY INORGANIC NITRATE: ROLE IN EXERCISE PHYSIOLOGY, CARDIOVASCULAR AND METABOLIC REGULATION

Filip Larsen

Karolinska Institutet

Stockholm 2011

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7457-397-8

ABSTRACT

Nitric oxide (NO) is a ubiquitous signaling molecule with a vast number of tasks in the body, including regulation of cardiovascular and metabolic function. A decreased bioavailability of NO is a central event in disorders such as hypertension and metabolic syndrome. NO is also important in the regulation of blood flow and metabolism during exercise. The production of NO has previously been thought to be under the exclusive control of the nitric oxide synthases (NOS) but this view is now being seriously challenged. Recent lines of research suggest the existence of an NO-synthase independent pathway in which the supposedly inert NO oxidation products nitrate (NO_3^-) and nitrite (NO_2^-) can be reduced back to NO in blood and tissues. An important additional source of nitrate is our everyday diet and certain vegetables are particularly rich in this anion. In this thesis the possibility that dietary derived nitrate is metabolized in vivo to form reactive nitrogen oxides with NO-like bioactivity has been explored.

It is shown that nitrate in amounts easily achieved via the diet, increases the systemic levels of nitrite and reduces blood pressure in healthy humans. Moreover, nitrate reduces whole body oxygen cost during submaximal and maximal exercise; a surprising effect involving improvement in mitochondrial efficiency and reduced expression of specific mitochondrial proteins regulating proton conductance. Alterations in the mitochondrial affinity for oxygen can explain this reduction in both submaximal and maximal oxygen consumption and predicts basal metabolic rate in humans. Finally, in mice lacking endothelial NO synthase, dietary supplementation with nitrate could reverse several features of the metabolic syndrome that develop in these animals. These studies demonstrate that dietary nitrate can fuel a nitrate-nitrite-NO pathway with important implications for cardiovascular and metabolic functions in health and disease.

LIST OF PUBLICATIONS

This thesis is based on the following papers; they will be referred to by their Roman numerals in the text.

I. **Effects of dietary nitrate on blood pressure in healthy volunteers.**
Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E.
N Engl J Med. 2006 Dec 28;355(26):2792-3.

II. **Effects of dietary nitrate on oxygen cost during exercise.**
Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B.
Acta Physiol (Oxf). 2007 Sep;191(1):59-66.

III. **Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise**.
Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B.
Free Radic Biol Med. 2010 Jan 15;48(2):342-7.

IV. **Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice.**
Carlström M, Larsen FJ, Nyström T, Hezel M, Borniquel S, Weitzberg E, Lundberg JO.
Proc Natl Acad Sci U S A. 2010 Oct 12;107(41):17716-20.

V. **Dietary inorganic nitrate improves mitochondrial efficiency in humans**
Larsen FJ, Schiffer TA, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E
Cell Metabolism. 2011 Feb 2;13(2):149-59.

VI. **Mitochondrial oxygen affinity predicts basal metabolic rate in humans**
Larsen FJ, Schiffer TA, Sahlin K, Ekblom B, Weitzberg E, Lundberg JO
FASEB J (accepted for publication).

CONTENTS

LIST OF ABBREVIATIONS

ADP	**Adenosine di phosphate**
ANT	**Adenylate nucleotide transporter**
ATP	**Adenosine tri phosphate**
BMR	**Basal metabolic rate**
COX	**Cytochrome c oxidase**
CS	**Citrate Synthase**
EDRF	**Endothelium-derived relaxing factor**
ETS	**Electron transport system**
FCCP	**Carbonyl cyanide 3-chlorophenylhydrazone**
HRmax	**Maximum heart rate**
HRR	**High Resolution Respirometry**
mtDNA	**Mitochondrial deoxyribonucleic acid**
nDNA	**Nuclear deoxyribonucleic acid**
NO	**Nitric oxide**
NOS	**Nitric oxide synthase**
sGC	**Soluble guanylyl cyclase**
cGMP	**Cyclic guanosine monophosphate**
PGC-1α	**Peroxisome proliferator-activated receptor gamma coactivator 1-alpha**
PVDF	**Polyvinylidene Fluoride**
RCR/RCI	**Respiratory control ratio/index**
RER	**Respiratory exchange ratio**
ROS	**Reactive oxygen species**
RNS	**Reactive nitrogen species**
NRF	**Nuclear respiratory factor**
TFAM	**Transcription factor A, mitochondrial**
UCP-3	**Uncoupling protein 3**
$\mathbf{VO_2}$	**Volume of consumed oxygen**
$\mathbf{VCO_2}$	**Volume of exhaled carbon dioxide**
$\mathbf{VO_2max}$	**Maximal oxygen consumption**
$\mathbf{P50_{mito}}$	**Oxygen tension were mitochondrial respiration is half-maximal**

INTRODUCTION

Physiology of nitric oxide

In a seminal paper published in 1980 Furchgott and Zawadzki showed that blood vessels release a vasodilating substance from the endothelium in response to a wide range of stimuli [1]. This compound was initially termed EDRF (endothelium-derived relaxing factor) and was subsequently identified to be nitric oxide (NO), a tiny free radical gas [2]. In 1998 Robert Furchgott, Louis Ignarro and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine for the discovery of endogenous NO production and its role as a signaling molecule in the cardiovascular system. NO is synthesized in the body by a family of enzymes known as the nitric oxide synthases (NOS). The NOSs catalyze the oxidation of L-arginine to L-citrulline and NO with oxygen as a co-substrate. These enzymes exist in three isoforms; neuronal (nNOS, NOS1), endothelial (eNOS, NOS3) and inducible (iNOS, NOS2).

NO exerts a vast number of signaling and regulatory functions in the body. In the circulation it has vasodilatory and anti-aggregatory properties. Primarily it acts as a second messenger in vasodilation which occurs via activation of soluble guanylyl cyclase (sGC), with resulting formation of cyclic guanosine mono phosphate (cGMP). cGMP then activates intracellular protein kinases ultimately resulting in vascular smooth muscle relaxation. The importance of NO in physiological regulation of vascular tone in mammals was early shown by the immediate increase in blood pressure that follows administration of a NOS inhibitor [3]. NO also has a number of effects that are independent of cGMP, one of these being its ability to competitively inhibit cytochrome *c* oxidase (COX) [4-6] the terminal enzyme in the mitochondrial electron transport system (ETS). Indeed, when the endogenous NO synthesis is blocked, blood pressure and tissue oxygen consumption increases [7]. Another proposed cGMP-independent function of NO and its reaction products is to facilitate reversible S-nitrosation of critical thiols in proteins, thereby regulating their function [8]. Moreover, the killing of bacteria, viruses, fungi and tumor cells by NO and its reaction products occurs mostly via cGMP–independent mechanisms [9].

For almost a century, nitroglycerin and other so called “organic nitrates” have been harnessed therapeutically against angina pectoris and high blood pressure [10]. In studies predating the discovery of endogenous NO generation this agent was showed to dilate

coronary arteries via the release of NO [11]. Today clinical applications of NO research include inhalation of NO to newborn babies with persistent pulmonary hypertension [12] and the use of exhaled NO as a marker of airway inflammation in asthmatics [13].

Production and consumption of NO

The levels of NO in the tissues are tightly regulated and determined by the balance between its production and consumption. The K_m values for oxygen (the oxygen concentration were NO production is half-maximal) is around 20 µM for eNOS, 130 µM for iNOS and 350 µM for nNOS [14] which is generally higher than the normal oxygen tension in the tissue that ranges from as low as 3 µM in contracting muscles [15] up to 60 µM [16] in some tissues under resting conditions. This means that the formation of NO from the enzymatic pathway is tightly regulated by the oxygen tension with more NO being generated at higher oxygen tensions.

NO has a very short half-life in vivo, ranging from milliseconds to a few seconds depending on the surrounding chemical milieu and metabolic state of the tissue where it is produced. Once formed, NO is rapidly oxidized to nitrite (NO_2^-), and eventually to nitrate (NO_3^-) which is considered the terminal oxidation product. A classical reaction is the rapid and effective oxidation of NO following its reaction with oxygenated hemoglobin in blood thereby forming methemoglobin and nitrate. The rate of NO consumption is also dependent on oxygen tension with faster consumption at higher oxygen concentrations [17]. As mentioned above, one proposed physiological function of NO is to modulate mitochondrial respiration via COX inhibition which in turn will increase the oxygen tension in the tissue. In this manner, excessive NO production can thus mimic hypoxia but oxygen levels are in fact higher than normal, leading to pathological production of reactive nitrogen species (RNS) and possibly irreversible modifications of proteins [18]. The chemical characteristics of NO also make this gas very reactive towards other free radical species, in particular superoxide (O_2^-) thereby forming peroxynitrite ($ONOO^-$). Peroxynitrite itself is also reactive and when protonated it can decompose to form nitrogen dioxide ($NO_2^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$) – both potent oxidants with potentially pathological consequences. The cytotoxic properties of NO and its reaction products is also utilized by the white blood cells and other cells in response to infections with bacteria, virus or parasites. The ability to stimulate sGC, inhibit mitochondrial respiration, the reactivity towards superoxide and the oxygen regulated half-life make NO very sensitive towards the metabolic condition and redox state of the tissue thus allowing it to act as a metabolic sensor and signaling molecule.

The nitrate-nitrite-NO pathway

Ever since the discovery of endogenous NO formation the anions nitrite and nitrate have been considered inert oxidation products and have merely been utilized by researchers as markers of NOS activity. However, recent lines of research have seriously challenged this view and evidence for a reverse pathway in which nitrate and nitrite are reduced back to NO is now emerging. The bioactivation of nitrate to NO involves a peculiar enterosalivary pathway. In humans and other mammals circulating nitrate originates from endogenous and dietary sources. This nitrate is actively absorbed by the salivary glands and excreted in saliva which has more than 10-fold higher nitrate levels than blood [19]. In the oral cavity parts of the nitrate is reduced to nitrite by symbiotic bacteria residing on the back of the tongue [20]. These facultative anaerobic bacteria respire by utilizing nitrate as a terminal electron acceptor. The bacterial conversion of nitrate to nitrite results in salivary nitrite levels of around 50-250 μM, i.e. thousand-fold higher than in plasma (50-250 nM). In the acidic gastric milieu a non-enzymatic reduction of salivary nitrite to NO occurs after protonation of the nitrite anion. In fact, this is the site where NOS-independent NO generation was first discovered [21-22]. Not all nitrite entering the stomach is reduced to NO as described above but much is actually absorbed intact into the blood stream as shown by an increase in plasma nitrite after oral nitrate intake [19, 23]. More than 1 liter of saliva is produced and swallowed each day and this pathway therefore constitutes a large part of the nitrite delivered to the circulation.

	Plasma nitrate	**Plasma nitrite**	**Saliva nitrate**	**Saliva nitrite**
Fasting	30 ± 4	0.123 ± 0.019	190 ± 30	100 ± 21
Nitrate intake	432 ± 44	0.229 ± 0.046	8200 ± 1000	700 ± 150

Table 1) Concentrations (μM) of nitrate and nitrite in plasma and saliva at fasting conditions and 30 minutes after nitrate intake (10 mg kg^{-1}). Adopted from [19].

Interestingly, during the past decade it has become clear that a number of enzymatic pathways exist in blood and tissues for the further one-electron reduction of nitrite to NO. These include deoxyhemoglobin in blood and also tissue proteins including deoxymyoglobin [24], neuroglobin [25], cytochrome c oxidase [26], xanthine oxidoreductase [27], aldehyde oxidase [28], and even NOS itself [27]. In addition, acidic non-enzymatic nitrite reduction, similar to that in the stomach, may also occur systemically and this reaction is greatly enhanced in the presence of reducing agents including vitamin C

and polyphenols [29]. In the circulation the nitrite reductase capacity of deoxyhemoglobin has been proposed as an allosteric-dependent and hypoxia sensitive regulating mechanism of microcirculatory blood flow [30]. The nitrate-nitrite-NO pathway might seem redundant when there is already three isoforms of NOS with high capacity for NO production. However, a fundamental difference between the two pathways is that the NOSs are oxygen dependent [14] while the nitrate-nitrite-NO pathway instead is greatly facilitated under hypoxia [31]. Thus, the latter pathway can be viewed as complementary to the NOS-dependent pathway and can operate also under conditions with low tissue oxygen tension such as during exercise or tissue ischemia. In fact, the low physiological oxygen tensions during muscular contractions [15] and the relatively high K_m of the NOSs for oxygen predicts that the enzymatic production of NO will be compromised during exercise. Under these conditions nitrite could possibly act as an alternative source of NO but it is important to bear in mind that besides NO, other potentially bioactive reactive nitrogen oxides might be formed from nitrite. These include nitros(yl)ation products and possibly also nitration products. Figure 1 is a schematic overview of the mammalian nitrate cycle and the biological targets for NO in the body.

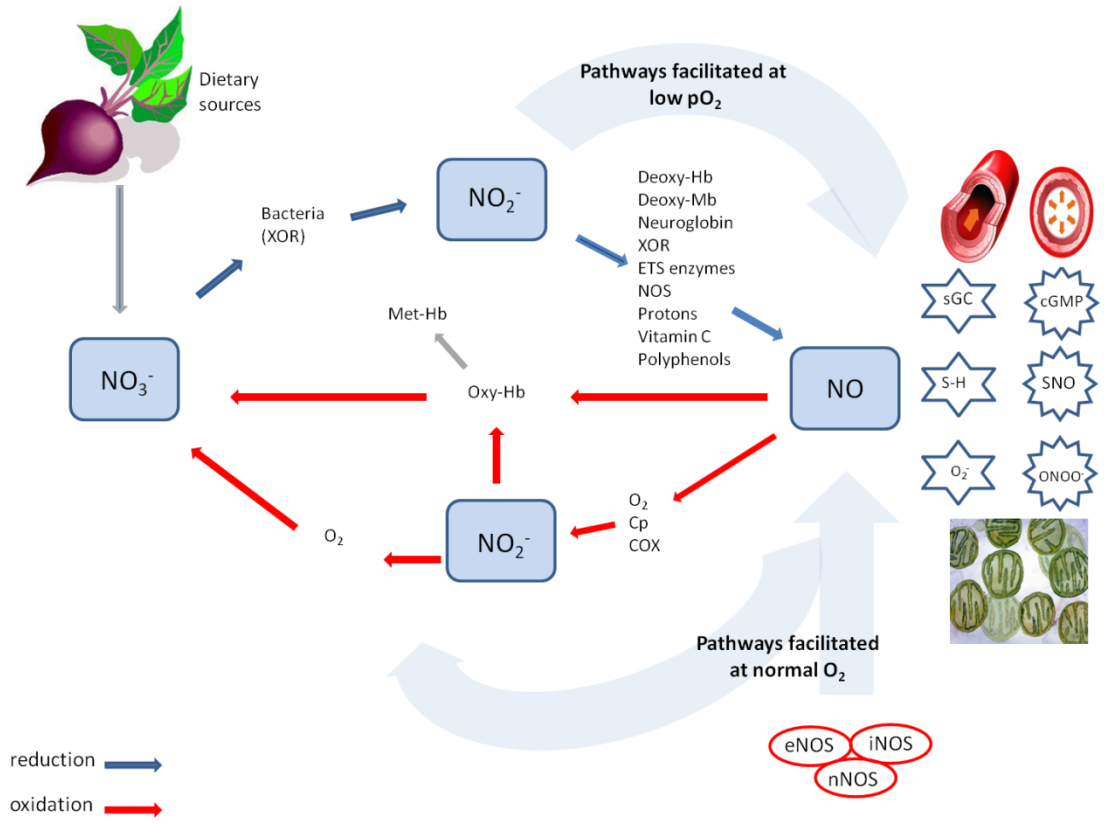

Figure 1) Overview of the mammalian NO-cycle. Nitrate originating from both dietary and endogenous sources can be reduced to nitrite by oral bacteria or to a lesser extent xathine oxidoreductase (XOR). The further reduction from nitrite to NO is facilitated at low oxygen tension and can be catalyzed by several mechanisms. Once formed, NO can be oxidized back to nitrite by simple autooxidation or enzymatically via ceruloplasmin (Cp) and COX. Nitrite also reacts quickly with oxyhemoglobin forming nitrate and methemoglobin. NO can have direct biological effects or act as a second-messenger by stimulation of sGC (soluble guanylyl cyclase) to produce cGMP which vasodilates blood vessels. Further, NO can react with thiols (S-H) forming s-nitrosothiols (SNO) with important regulatory functions. NO interaction with mitochondrial enzymes may result in superoxide formation and the reaction products peroxynitrite ($ONOO^-$) and hydrogenperoxide (H_2O_2) can have signaling functions in the cell.

Dietary sources of nitrate and nitrite

Besides the NOSs, the diet constitutes a major source of nitrate since it is found in a rather wide range of food sources. Vegetables are especially rich in nitrate with green leafy vegetables such as spinach, lettuce and beetroot being the richest known sources containing up to 740 mg 100 g^{-1} fresh weight [32]. In contrast, most fruits are low in nitrate. Unfortunately, vegetables and fruits are often lumped together in epidemiological studies investigating the health effects of our diet. The levels of nitrite are low in most vegetables but higher in cured meats such as bacon and ham where it is added as a preservative [32]. Under normal conditions the contribution of nitrate and nitrite from dietary vs endogenous sources is roughly equal but with a diet rich in vegetables this source becomes dominant [32]. Conversely, with massive activation of NOS such as in systemic inflammation, the contribution of nitrate from the NOS pathways increases dramatically [33]. Plasma levels of nitrate and nitrite can thus be influenced either by the activity of the NOSs or by adjusting the dietary intake of these two anions. Another possible means of manipulating plasma nitrite levels is to disrupt the enterosalivary pathway, either by using an antibacterial mouthwash or by spitting. Both these procedures have been used experimentally and they strongly attenuate the increase in nitrite seen after nitrate intake [19] and possibly also reduce basal nitrite levels. In aggregate, the systemic levels of nitrate and nitrite are highly dependent on our diet, and the fact that they can be considered as a major pool of potential NO bioactivity in the body raises the question concerning the nutritional value of theses anions.

Is dietary nitrate carcinogenic?

For more than half a century dietary nitrate has been considered a carcinogenic substance and a toxic residue in our food and water and the content is therefore strictly regulated in drinking water and fertilizers. The supposed carcinogenic mechanism is nitrite-dependent formation of nitrosating agents which can react with dietary amines, forming nitrosamines, substances with known carcinogenic properties [34]. However, despite extensive research no casual link between dietary nitrate intake and gastric cancer in humans has been found [35-36]. Another concern regarding nitrate in food and drinking water is the risk of methemoglobinemia in small children (blue baby syndrome). This can occur when nitrate is reduced to nitrite by bacteria in contaminated food and drinking water and toxic amounts of nitrite are ingested. However, extremely few cases have been reported during the last decades. Excessive nitrate intake can inhibit uptake of iodide by the thyroid gland. Indeed, high nitrate exposure in humans has been related to hypertrophy of the thyroid but without affecting iodine levels [37]. Recently, an association between nitrate levels in drinking

water and thyroid cancer was found in older women [38]. Some precautions are warranted when extrapolating nitrate levels in drinking water to cancer risks since nitrate in the water can be indicative of bad water quality in general. Further, only a small fraction of total daily nitrate exposure originates from water, the majority of nitrate (>80%) is derived from vegetables [9]. In general a diet rich in vegetables is associated with reduced risk of cancer [39-41]. Despite the apparent lack of evidence for a carcinogenic role of dietary-derived nitrate, research is still lacking regarding the long-term safety of nitrate salts, such as those used in this thesis.

NO and the metabolic syndrome

The metabolic syndrome is a cluster of medical conditions that increase the risk of developing diabetes and heart disease. The prevalence is rapidly increasing and around 20% of the population in the United States is affected with increased blood pressure, glucose intolerance, dyslipidemia and central obesity as the diagnostic criteria [42]. Interestingly, a genetic polymorphism that is associated with a defect in the endogenous production of NO has been proposed as a candidate for the underlying molecular mechanism behind the development of the metabolic syndrome [43-44]. In further support of the hypothesis that NO deficiency is important in the progression of this syndrome, it has been shown that mice lacking the eNOS gene ($eNOS^{-/-}$) develop hypertension, hyperlipidemia and insulin resistance as they age [45]. NO is important in multiple functions that can explain why NO deficiency leads to the development of these symptoms. NO is directly implicated in skeletal muscle glucose uptake [46-47] and mediates insulin secretion from beta-cells [48]. Further, NO has been shown to trigger mitochondrial biogenesis [49] which is important to maintain the respiratory capacity in the tissue, a function that is often reduced in diabetes [50]. A change in diet is often suggested to patients with metabolic syndrome, diabetes or hypertension as a primary therapeutic intervention. A diet rich in fruit and vegetables, such as the Mediterranean diet, is considered beneficial under these circumstances [51]. Interestingly, this will provide high amounts of dietary nitrate and this fact triggered the studies on blood pressure and metabolic syndrome in this thesis.

Regulation of oxygen consumption

A considerable part of this thesis is concentrated around the effects of dietary nitrate on various exercise parameters, in particular oxygen consumption. It therefore seems reasonable to discuss some basic exercise physiology.

Oxygen consumption during submaximal exercise

The oxygen consumption during submaximal work rates is dictated by the external work rate and the efficiency of energy transduction. During submaximal exercise on a cycle ergometer, oxygen uptake under steady-state conditions is an almost linear function of work rate and at any given level one finds very small inter- and intra-individual variations, regardless of training status [52], age or diet [53]. During such tests energy expenditure is most often measured by indirect calorimetry (oxygen consumption and carbon dioxide release), assuming that metabolic heat production is reflected by the amount of oxygen consumed. Gross efficiency (the ratio of mechanical work output to the metabolic energy input) during cycling is around 18-23 % in humans [54] with small inter-individual differences. The efficiency is dictated by the amount of slow-twitch, oxidative muscle fibers [55] and also the abundance of uncoupling protein 3 (UCP-3) expression [55], but physical training status does not seem to affect cycling efficiency [56]. On the mitochondrial level efficiency is often expressed as the amount of ATP produced per oxygen consumed on a molar basis (P/O ratio) [52, 56-57]. However, recent work indicates that mitochondrial P/O ratios *in vivo* are quite far below the estimated maximal 2.5 *in vitro* [55, 58-59] which implies that mitochondria operate below maximal efficiency. Indeed, to obey the second law of thermodynamics all systems operating out of equilibrium must be inefficient/uncoupled to some degree. The rather low gross efficiency could theoretically be explained by the degree of mitochondrial coupling but to date experimental evidence for a correlation between mitochondrial efficiency and muscular efficiency is lacking [55]. Part of the ATP produced in the mitochondria is used in the contraction of the muscular actin-myosin filaments to generate mechanical work. However, the contractile efficiency is only around 60% [60] and thus constitutes yet another route of energy loss.

Oxygen consumption during maximal exercise

In contrast to oxygen uptake during submaximal work, the maximal oxygen consumption (VO_2max) is not set by the external work rate but is limited by the subjects' maximal ability to transport oxygen. The factor(s) that limit the maximal ability to extract oxygen from the air and its transport has been heavily disputed over (for review see [61]). Evidence now lean towards that cardiac output and central oxygen delivery is the major bottleneck in

the respiratory cascade, at least during work involving large muscle groups. This is illustrated by the change in VO_2max when inducing anemia (decreased VO_2max) or polycythemia (increased VO_2max) to manipulate the oxygen carrying capacity of the blood [62]. Further, combined arm and leg exercise does not [63-64] or only marginally [65-67] increase VO_2max compared to leg exercise alone, although the peripheral respiratory capacity has increased substantially. These and other human data [68] that show an excess in mitochondrial capacity predict that intracellular oxygen concentration will decrease to critically low levels during exercise. Indeed, oxygen tensions as low as 0.3-0.4 kPa (3-4 µM) are found in working muscles [15, 69] rendering the ability of mitochondria to maintain respiration at these low oxygen tensions crucial to avoid severe oxygen limitation of respiration. The mitochondrial respiration rate ($j'O_2$) is a hyperbolic function of oxygen tension:

$$j'O_2 = \frac{pO_2}{pO_2 + p50_{mito}}$$

where pO_2 is the average intracellular oxygen tension and $p50_{mito}$ is the oxygen tension were mitochondrial respiration proceeds at 50% of the oxygen saturated maximum rate. This equation explains the influence of $p50_{mito}$ on mitochondrial respiration rate. A wide range of $p50_{mito}$ values can be found in the literature, from as low as 0.002 kPa [70] to 0.3 kPa [71]. If the $p50_{mito}$ was to be 0.002 kPa, oxygen availability would not exert any control over respiration. On the contrary, $p50_{mito}$-values reaching 0.25 kPa would indicate that mitochondrial respiration would be half-inhibited already at normal physiological oxygen concentration and there would be little room for normal perturbations such as inhibition by NO or decreased respiration due to oxidative damage. Recently, advances in mitochondrial respiration research, through the introduction of High Resolution Respirometry (HRR), have allowed more precise measurements of $p50_{mito.}$ Using this technique, $p50_{mito}$ in rat heart and liver mitochondria is around 0.03-0.06 kPa [72]. Considering an oxygen tension of 0.4 kPa in working muscle, this means that mitochondrial respiration only reach approximately 90% of its maximum rate under these conditions. In the presence of physiological concentrations of NO the $p50_{mito}$ can be twice as high [73] due to the competitive inhibition of COX [74]. Together these results imply that even if the pumping capacity of the heart is the major determinant and limiting factor of VO_2max, mitochondrial oxygen affinity ($p50_{mito}$) can have a small but important regulatory role during maximal exercise.

Control of mitochondrial respiration by NO

One of the primary regulatory effects of NO is its ability to competitively and reversibly bind to COX and thereby inhibiting mitochondrial respiration. This was first described by applying NO-donors to isolated mitochondria [4-6] and later NOS-inhibition was shown to be associated with increased oxygen consumption in resting dog muscles [75], a finding subsequently reproduced in several studies [76-78]. However, the effects of NO-donors on whole body metabolism and oxygen consumption are more divergent, with studies showing inhibitory [79-80] or no effects [81]. Even though it has been unequivocally shown that NO can inhibit COX, this inhibition does not necessarily affect mitochondrial respiration since there is an overcapacity of COX relative to the flux of the electron transport system (ETS) [82-83]. NO can thus inhibit COX without reducing respiration up to the point where COX becomes rate limiting. The biological advantage of this apparent excess capacity of COX is at present unknown but there is evidence that a high COX activity is needed to keep mitochondrial oxygen affinity at a sufficiently high level (low $p50_{mito}$) [83], while others argue that it is needed to avoid severe inhibition by NO under normal physiological conditions [82]. Given the excess of peripheral mitochondrial respiratory capacity over oxygen delivery from the heart and the excess of COX over other parts of the ETS, COX has to be severely inhibited before whole body oxygen consumption declines. In fact, even when isolated mitochondria are stimulated to full state 3 respiration only a fraction of the total COX capacity is utilized [84-85]. Still, studies that block endogenous NO-synthesis have repeatedly shown an increase in oxygen consumption both at the tissue and whole body level [75-78]. This indicates that physiological NO levels of around 20 nM [86] indeed have an impact on tissue oxygen consumption. An O_2/NO ratio of around 500 has been proposed to attain half-inhibition of COX [73]. This would imply that at 10 µM oxygen, which is a physiological tissue oxygen tension, COX is already half-inhibited by NO. During increased metabolic demand oxygen tension can be as low as 3-4 µM [15, 69] and inhibition of NO is further increased. However, the excess of COX over other complexes in the ETS has been estimated to be eight-fold [87] allowing for severe inhibition of COX before mitochondrial respiration decreases.

Another hypothesis on how nano molar concentrations of NO could influence oxygen consumption suggests that NO improves oxidative phosphorylation efficiency [88] indirectly via COX inhibition. COX transfer electrons to oxygen, simultaneously protons are pumped across the inner mitochondrial membrane. However, electrons can pass through the COX protein without pumping of protons [89-91], a phenomenon termed "proton slippage". When COX is partially inhibited by NO and respiration slightly

decreases, there is evidence that ATP production is maintained to a larger degree than oxygen consumption, thereby increasing the P/O ratio. This effect has been attributed to a reduction in proton slippage [88] which is supported by the fact that inhibition of endogenous NO-production increases oxygen consumption without changes in ATP production [92].

Mitochondrial efficiency and proton leak

As mentioned above there are several indications that mitochondria operate under less than maximal P/O ratios under physiological conditions. First, P/O ratios typically decrease as respiration decreases from state 3 (respiration with substrates and ADP) to state 4 (respiration after ADP depletion) [55, 58, 93]. Given the excess mitochondrial respiratory capacity, physiological respiration is more close to state 4 than state 3, especially at a lower metabolic demand. Further, P/O ratios from *in vivo* measurements in humans indicate that some muscles exhibit mild uncoupling [94]. In its broadest sense uncoupling refers to protons that are pumped over the inner mitochondrial membrane but are unrelated to ATP-synthesis. The cause of uncoupling is relatively unknown but several putative mechanistic sites of proton leak have been proposed such as through the adenylate nucleotide transporter (ANT) [95-96], loss via metabolite transport through the UCPs [97] and also by unspecific permeability through the inner membrane [98]. Interestingly, UCP-1, the major metabolic uncoupler previously only found in rodents, has recently been found in small amounts also in humans [99] were it seems to be metabolically active [100] and can be important in thermogenesis and weight control. In humans, UCP-3 is found uniquely in skeletal muscle but its role in uncoupling is still debated. Another possible pathway of decreased coupling is by slip of the proton pumps as mentioned earlier, i.e. electrons can be transferred through a proton pumping complex without proton translocation. Slip seems to occur in all proton pumps at high membrane potential but is probably only physiologically significant in COX [101] .

AIMS

The general objective of this thesis was to investigate metabolic and circulatory effects of inorganic nitrate in healthy humans as well as in rodents. New questions developed over time and the specific aims were:

- To investigate the effect of dietary nitrate supplementation on resting blood pressure and blood pressure in response to exercise in healthy volunteers (study I and III).
- To investigate the metabolic and circulatory response to exercise after supplementation with dietary nitrate in healthy subjects (study II and III).
- To investigate if features of the metabolic syndrome that develops in eNOS-deficient mice would be affected by dietary nitrate supplementation (Study IV).
- To explore the effects of dietary nitrate on human skeletal muscle mitochondrial function in relation to whole body oxygen consumption during exercise (Study V).
- To examine the relationship between mitochondrial oxygen affinity and different degrees of metabolic efficiency at rest and during exercise (Study VI).

MATERIALS AND METHODS

A summary of the methods used in this project is presented below. For a more detailed description the reader is referred to the individual papers.

Subjects

In study I, II, III, V and VI healthy volunteers were enrolled. In the initial screening subjects were excluded if they were regular smokers, followed a vegetarian diet, were on chronic medication or had any other chronic illness or disability. The subjects in study II were well-trained young men, predominantly cyclists and triathletes. The subjects in study III, V and VI were healthy and active but not necessarily competitive athletes.

Animals

In study IV female eNOS-deficient, wild-type, and nNOS-deficient mice were obtained from Jackson Laboratories. Animals were randomly assigned to placebo and nitrate to ensure that each group had similar average age and weight. Sodium nitrate (85 mg L^{-1}) was added to the drinking water during 8 to 10 weeks. For more detailed information on animal strains, housing and treatment see [102].

Nitrate supplementation and dietary restrictions

For three days (two days in study III) before the tests, subjects were instructed to adhere to a diet with low nitrate content. This diet excluded all vegetables, cured meats, tea and alcohol-containing products which eliminate all food stuff with medium or high nitrate content but a small amount of nitrate intake still remains from drinking water and foods with lower nitrate content. In conjunction with these dietary restrictions, subjects were given 0.1 mmol kg^{-1} day^{-1} sodium nitrate or an equimolar amount sodium chloride (placebo) divided in three doses per day.

Exercise protocols

In study II an incremental exercise protocol was used with five submaximal workloads each lasting five minutes starting at approximately 45% and ending at 85% of VO_2peak. There was no rest period between the work rates. After the submaximal test, a maximal exercise test was initiated starting at approximately 90% of VO_2peak and increased every minute until exhaustion. In study III a maximal exercise test similar to the one in study II was used, but using combined arm and leg exercise, see study III for further details. In

study V and VI a single work rate was used were subjects cycled for approximately ten minutes to determine metabolic demand and circulatory parameters.

Determination of VO_2 and metabolic parameters

Oxygen consumption, pulmonary ventilation and CO_2 output (V_{CO_2}) were measured using an online metabolic gas analyser (AMIS 2001, Odense, Denmark in study II and III, Jaeger Oxycon Pro, Hoechberg, Germany in study V and VI).The AMIS 2001 gas analyzer was connected to a flow meter which the subjects breathed through via a mouthpiece and a plastic tube. The Jeager was used in conjunction with a face mask directly connected to a flow meter. In study III the Douglas bag technique was used together with the AMIS 2001 as described [63]. The respiratory exchange ratio was calculated as the volume of exhaled carbon dioxide divided by the volume of oxygen uptake (VCO_2/VO_2). The basal metabolic rate (BMR) was measured after an overnight fast (at least 12 hours) and after at least 36 hours of abstinence from physical training or other exhaustive activities. The subjects were allowed to rest on a bed in a quiet thermo neutral room for 30 minutes and then BMR was measured using the Jeager Oxycon Pro system described above. A plastic hood was placed over the head of the subject and ventilation was controlled by a computerized turbine and set between 25-50 L min^{-1}. The lowest steady oxygen uptake recorded for approximately 10 minutes was used as the BMR.

Measurements of blood pressure

Blood pressure was measured in study I, II and III with a stethoscope and an inflatable sphygmomanometer, placed over the brachial artery. The first Korotkoff sound was used as systolic pressure while the fifth phase was interpreted as diastolic pressure. Blood pressure measurements were made with the subjects lying in the supine position. The resting blood pressure measurements were initiated after the subjects had been resting in a quiet room for at least 30 minutes.

Calculation of energy expenditure and efficiency during cycling

Energy expenditure was calculated from gas exchange data using the equation by Brouwer [103] in study II and V. The gross efficiency of energy conversion was calculated as the mechanical power output divided by the metabolic energy input. Delta efficiency is a measure of the increase in metabolic energy input for a given increase in mechanical work output and was calculated as the slope of the linear regression line over the four lowest workloads according to Gaesser and Brooks [54]. Net efficiency is simply the mechanical

workload divided by (the basal metabolic rate subtracted from the total energy expenditure) [54].

Muscle biopsies

After anaesthesia (Lidocain without epinephrine) of the skin and muscle fascia, a small incision was made at the midsection of the *Vastus Lateralis*. Muscle biopsies were obtained using a chonchotome at a depth of 1-2 cm below the fascia. Biopsies were taken in a randomized fashion from the left or right leg when subjects had been treated with placebo or nitrate. Parts of the biopsies were immediately snap frozen in liquid nitrogen (-196°C) and stored in -80°C for later analysis of enzymatic activity or protein expression. The rest of the muscle tissue was placed in mitochondrial isolation medium (see below) and soon thereafter the isolation of mitochondria was initiated.

Isolation of mitochondria

The part of the muscle tissue designated for mitochondrial isolation was first weighed and then cut for five minutes in ice-cold isolation medium (Sucrose 100 mM, KCl 100 mM, Tris-HCl 50 mM, KH2PO4 1mM, EGTA 100 µM, BSA 0.1%), final pH was set to 7.4. After cutting, the homogenate was rinsed again in 1 ml isolation medium and the supernatant removed. 1 ml of isolation medium containing 0.2 mg ml^{-1} bacterial protease was added to the homogenate. The homogenate was incubated in this medium for a total of two minutes with 30 seconds of gentle shaking alternating with 30 seconds on ice. The homogenate was then transferred to a water-cooled glass jacket and homogenized with a hand held electrically driven drill (80 rpm) and immediately transferred to a falcon tube containing 3 ml isolation medium and subsequentially centrifuged at 700 *g* and 4° C for ten minutes. After removing the pellet the suspension was again centrifuged at 10000 *g* and 4° C. The pellet containing the mitochondria was carefully washed and then resuspended in 0.65 ml isolation medium. After centrifugation at 7000 *g* for 5 minutes the pellet was dissolved in 0.6 µl preservation medium (EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, K-lactobionate 60 mM, Taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, Sucrose 110 mM, BSA 1 g/l Histidine 20 mM, Vitamin E succinate 20 µM, Glutathion 3 mM, Leupeptine 1 µM, Glutamate 2 mM, Malate 2 mM, Mg-ATP 2 mM) per mg weight original tissue.

Mitochondrial respiration

All mitochondrial experiments presented in study V and VI were performed in a two-channel respirometer (Oroboros Oxygraph, Paar, Graz, Austria) with the glass chamber volume set to 2 ml. PVDF magnetic stirrers and stoppers were used in conjugation with

rubber sealing to minimize oxygen back diffusion into the chamber [104]. Data were collected with one second intervals and averaged over 40 seconds. The medium in the respiration chamber was MiR05 containing (EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, K-lactobionate 60 mM, Taurine 20 mM, KH_2P0_4 10 mM, HEPES 20 mM, Sucrose 110 mM, BSA 1 g/l). All experiments were performed at 37° C; speed of the magnetic stirrers was set to 750 rpm. Time constants for complete mixing in the chamber were calculated by briefly stopping and starting the stirrers. Oxygen consumption and zero-drift of the oxygen electrode were calculated using DatLab 2 and DatLab 4 software (Oroboros, Paar, Graaz, Austria). Diffusion of oxygen into the chamber was calculated as a linear function of oxygen tension in a separate background experiment using at least five different oxygen tensions. This background experiment were performed each time the membranes of the polarographic oxygen sensor were changed or when otherwise considered necessary. The $p50_{mito}$ was calculated with DatLab 2 [105]. The term LEAK-respiration is used for the respiratory rate when mitochondria are exposed to saturating concentrations of respiratory substrates but in the absence of adenylates.

When ADP is added in saturating amounts, respiration increases several-fold and is termed state 3 respiration. When all ADP is phosphorylated to ATP, respiration enters state 4. This state is driven by proton leakage and ATPase activity. The uncoupled state or "state 3u" is achieved by titration with FCCP. In human mitochondria an increase in respiration rate is observed when adding FCCP, up to an optimal concentration where after respiration is inhibited.

P/O ratio

In study V we measured the P/O ratio in isolated mitochondria at constant infusion of non-saturating ADP-levels (modified from [58]) using a microdialysis pump (CMA 100, Solna, Sweden) through high-pressure hosing. Before starting the ADP-infusion, 2 mM ATP was added to the medium. ADP was infused at a rate corresponding to approximately 50% of maximal state 3 respiration. Infusion started at an oxygen pressure of approximately 10 kPa and was determined before oxygen pressure reached 1 kPa. The effective P/O ratio was calculated as the rate of infused ADP divided by the oxygen consumed during steady state at the last minutes of infusion. Correction was made for the amount of oxygen added to the respiration medium by the infused ADP solution. ADP concentrations were verified by spectrophotometry.

Estimation of thermodynamic coupling

Thermodynamic coupling (q) can be estimated using the equation:

$$q=\sqrt{1-\left(\frac{\text{Static head}}{\text{State 3u}}\right)}$$

In mitochondrial respiration static head responds to state 4 respiration when the ANT protein is blocked by atractyloside. The uncoupled "state 3u" is achieved by a titration protocol with FCCP. At optimal concentration, the membrane potential is collapsed and the mechanism that couples oxidation to phosphorylation disappears. Therefore, in the uncoupled state, the "friction" of producing ATP vanishes and mitochondrial respiration reaches a maximum. The q-value is then a measure of the degree of thermodynamic coupling with values ranging from 0.88-0.97 in different rat organs [106].

Western blotting

Freeze-dried muscle homogenates were dissolved in Laemmli sample buffer and denaturated by heating to 95º C for 10 minutes. Protein concentration of the homogenate was determined spectrophotometrically and 30 µg of protein was pipetted into each well on 12% polyacrylamide gels. The proteins were then separated by SDS-PAGE for 60 minutes. The separated proteins were transferred to a PVDF membrane at 10 V for 60 minutes and then blocked in TBS using 5% non-fat milk. Polyclonal antibodies against UCP3 (Chemicon AB3046), diluted 1: 500 and (ANT-1, Q-18, No.sc-9300, Santa Cruz) against ANT diluted 1: 200 were added to the solution and membranes were incubated over night. The membranes were then washed and again incubated with secondary antibody goat antirabbit (IgG-HRP, NO.sc-2030 Santa Cruz). The membrane was once again washed and a chemiluminescence detection marker was added. The optical density of the bands was quantified using Molecular Analyst 1.5 (Bio-Rad).

Rt-PCR

In study IV gene expression of PGC-1α was measured in liver and muscle samples. In study V gene expression of COX IV, TFAM and PGC-1α was measured in human muscle biopsies. RNA was extracted from frozen tissues with a power homogenizer (KEBO-lab) and TRIzol reagent. One µg of total RNA was reverse transcribed using M-MLV reverse transcriptase. Relative expression levels of each gene were determined by real time PCR in a 7900 sequence-detection system (Applied Biosystems). In study V nuclear and

mitochondrial DNA was extracted with DNAesy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. The mitochondrial DNA and nuclear DNA quantification was carried out by multiplex real-time PCR, using the mtDNA qkit (Genemore Italy srl, Modena, Italy). The signal derived from the probes for mtDNA and nDNA was detected with FAM and TexasRed filters, respectively. An ABI PRISM 7500 Sequence Detector (Applied Biosystems) was used for the amplification and detection.

IPGTT

In study IV animals were subjected to an intraperitoneal glucose tolerance test (IPGTT). After an overnight fast, animals were weighed and a bolus of glucose (30% in H_2O, 2 g kg^{-1}) was injected into the intraperitoneal cavity. Blood samples were obtained from the tip of the tail at 0, 15, 30, 60, and 120 minutes post-injection. Blood glucose levels were determined with a hand-held glucose meter (Glucocard X-SENSOR; OneMed).

Visceral fat, body weight and triglycerides

During the seven week treatment period mice were weighed once a week. At the termination experiment, all visceral abdominal adipose tissue was removed manually and weighed. Triglycerides in blood were determined using a commercial kit (Cayman Chemical).

Analysis of enzymatic activity

Enzymatic activity of CS was determined in homogenates from freeze-dried biopsies and isolated mitochondrial samples at 25° C as previously described [107]. COX activity was determined using Cytochrome *C* Oxidase assay kit from Sigma (CYTOCOX1) at 25° C according to the manufacturers' instructions.

Chemiluminescence assay for nitrogen oxides

Blood samples were collected in EDTA tubes and immediately centrifuged to avoid autooxidation of nitrite. Analysis of nitrite, nitrate, and nitros(yl)ation products were determined by a chemiluminescence assay after reductive cleavage and quantification of the NO released into the gas phase [19]. In short, NO reacts with ozone (O_3) which yields nitrogen dioxide (NO_2). When NO_2 in the electronically excited state ($^*NO_2$) returns to its ground state light is emitted and can be quantified by a photomultiplier. The samples were injected into a highly reducing solution in a micro reaction chamber. The condenser jacket temperature was controlled by a continuous flow of cold water while the temperature of the heating jacket was controlled by a continuous flow of warm water regulated by a constant-

temperature circulating bath. A constant, controlled flow of nitrogen was used as the carrier gas of NO. The resulting NO signal was reported as the area under the curve. Calibration curves were obtained by standard solutions of nitrate or nitrite in ultrapure water. When analyzing nitrite the reducing solution consisted of potassium iodide (KI) 45 mM and iodine (I_2) 10 mM, in acetic acid. The solution was kept at 56° C and continuously bubbled with nitrogen gas. Nitrite measurements were performed by injection of sample (50-100 µl) into the reducing solution. The amount of nitrite was quantified by simple subtraction of the peak area of sample aliquots pretreated with sulfanilamide from that of untreated aliquots. Nitrate was reduced to NO using a solution of vanadium (III) chloride in a saturated solution of hydrochloric acid at 95° C. This reducing solution will also convert nitrite to NO, so the amount of nitrite measured first was subtracted from the nitrate concentration. To avoid foaming, the samples were deproteinized prior to analysis with three times as much cold ethanol as plasma.

cGMP assay

Plasma concentrations of cGMP were analyzed with a commercial available ELISA-kit (Bio-track EIA-System, Amersham). Samples were prepared according to the manufacturers' instructions.

Methodological precision

The mitochondrial experiments in study V and VI were performed with High Resolution Respirometry, a method developed to improve the resolution of mitochondrial experiments. Relatively inert materials are chosen in the respiration chamber, stoppers and magnetic stirrers. Oxygen consumption of the electrode, diffusion of oxygen into the chamber, oxygen solubility in the medium and electrode drift are measured and corrected for. Temperature is controlled down to ±0.01° C. These precautions increases the resolution of the measurements but simultaneously a reduction of noise might reveal larger variations between different experiments. In Table 2 data are presented on the variability between two experiments performed at identical assay conditions but in different chambers and at slightly different times after isolation but from the same mitochondrial suspension. Coefficients of variation (CV) is calculated from:

$CV=100\ (SD_{n1\text{-}n2}/X_{n1n2})$

$SD_{n1\text{-}n2}$ is the standard deviation of the difference of a number of duplicate experiments, X_{n1n2} is the mean value of all those experiments. As a reference, *in vivo* data on submaximal and maximal oxygen consumption and heart rates are presented. These data are a sample of recent exercise tests (to be published) from our laboratory were subjects

perform identical submaximal and maximal exercise testing at two different days but under standardized laboratory conditions.

	Number of observations	*CV*
LEAK respiration	25	24.8 %
State 4 respiration	24	18.9 %
State 3 respiration	33	7.8 %
P/O ratio	23	10.6 %
$p50_{mito}$	24	12.5 %
Submaximal VO_2 cycling	20	3.6 %
VO_2max	40	2.9 %
HRmax	40	1.3 %

Table 2) Coefficients of variation between two separate observations of mitochondrial parameters measured in isolated mitochondria and whole-body physiological parameters measured during exercise.

In the mitochondrial experiments the CV is apparently a function of respiration rate with the lower respiration rates in LEAK and state 4 yielding a higher signal to noise ratio and thus higher CV. The CV for VO_2max and HRmax are very low and these can probably be considered two of the most stable parameters we can measure in biological systems.

Statistics

Overall results are expressed as mean ± S.E.M. In study II mean ± S.D was used. P-values <0.05 was considered significant. Student's paired t-test was used to compare single outcomes of placebo and nitrate supplementation in normally distributed data, otherwise Wilcoxon signed-rank test was used. Bonferroni correction was applied when multiple comparisons were made. Pearson r was used in correlation analysis. Data normality distribution was analyzed with D'Agostino and Pearson omnibus normality test. When multiple parameters were analysed two-way ANOVA with repeated measures were used. Dose-response experiments were analyzed with one-way ANOVA.

RESULTS AND COMMENTS

Plasma levels of nitrate and nitrite in the human studies.

After nitrate treatment, plasma concentrations of both nitrate and nitrite increased significantly in all studies (see Table 3). This finding confirms the effective absorption of nitrate and conversion to nitrite.

	TREATMENT	**STUDY I (N=17)**	**STUDY II (N=9)**	**STUDY III (N=9)**	**STUDY V (N=14)**
Nitrate (µM)	Placebo	26±11	27 ± 6.9	17 ± 3.0	27 ± 2.6
Nitrate (µM)	Nitrate	178±51*	182 ± 55*	230 ± 31*	169 ± 18*
Nitrite (nM)	Placebo	138±38	124 ± 28	61 ± 11	35 ± 7
Nitrite (nM)	Nitrate	219±105*	226 ± 87*	142 ± 35*	163 ± 29*

Table 3) Plasma concentrations of nitrate increased substantially after nitrate intake in all individuals. One hour after nitrate intake nitrite was elevated which confirms the salivary conversion of nitrate to nitrite. * $p<0.05$ versus placebo treatment. Values are mean ± S.E.M.

Effects of inorganic nitrate on cGMP-levels

We measured plasma levels of cGMP in study II, III, IV and V. In study IV we also measured cGMP in the tissue. The overall conclusion is that nitrate supplementation does not increase cGMP neither in blood nor in the tissue. If dietary nitrate does become reduced to NO, it is sound to expect that this NO would stimulate sGC to produce cGMP. However, the absence of effect on cGMP should not be interpreted as evidence that NO-production is not augmented by nitrate. Recently Kapil *et al.* found increased levels of cGMP in blood after administration of inorganic nitrate [108]. The sensitivity of the assay and

standardization of the blood sampling procedure is somewhat problematic. The CV of two measurements from the same subject on separate days is 72%, therefore small changes in cGMP are not readily detectable unless using a large number of subjects. Despite this, we cannot exclude that the nitrite ion itself or other nitrogen species exerts biological effects similar to NO or that the effects are mediated by cGMP-independent mechanisms.

Effects of inorganic nitrate on blood pressure

At the time of study I it was unknown if dietary nitrate could have any NO-like effects and nitrate was still considered to be virtually inert. If dietary nitrate is metabolised to appreciable amounts of NO systemically one would expect vasodilation to occur which in turn could affect blood pressure. Indeed, in study I resting diastolic blood pressure was reduced by 3.7 mm Hg (p=<0.02) and mean blood pressure was reduced by 3.2 mm Hg (p=0.03) after a three-day intervention with sodium nitrate (0.1 mmol kg^{-1} day^{-1}). Part of these data origins from study II. In study III we did not see any difference in resting blood pressures, (systolic 109 ± 5, diastolic 70 ± 2 mm Hg and systolic 109 ± 3, diastolic 69 ± 2 mm Hg in the placebo and nitrate group, respectively). However, in the period immediately after exercise the diastolic blood pressure decreased from 69 ± 2 to 62 ± 3 mm Hg in the nitrate group (p=<0.05), an effect that was not observed in the placebo group. The discrepancy in results between study I and II vs study III is unknown but nitrate administration was three days in study I and II but only two days in study III. Also, measurement of resting blood pressure was done only 45 minutes after the last nitrate dose in study III (one hour in study I and II) which in subsequent studies turned out not to be enough for the maximum conversion of nitrate to nitrite [23]. In study IV blood pressure was measured by telemetry in rats that received nitrate (0.1 mmol kg^{-1} d^{-1}) in the drinking water for 8 weeks. Mean arterial blood pressure measured over 72 hours was approximately 3 ±mm Hg lower in rats receiving nitrate compared to placebo (p<0.05). When rats were given L-NAME (1 g L^{-1}) in the drinking water, blood pressure increased significantly in both groups but still remained lower in the nitrate group. For unknown reasons this increase was delayed by 12 hours in the nitrate group.

Treatment of eNOS deficient mice with dietary nitrate

As they age, $eNOS^{-/-}$ mice develop insulin resistance, high blood pressure, elevated triglyceride levels and visceral fat accumulation, features also present in the human metabolic syndrome. When these mice were fed nitrate chronically in the drinking water, the disturbed glucose homeostasis was essentially reversed (see Figure 2). Chronic feeding with nitrate was associated with elevated levels of nitros(yl)ation products (RXNO)

including S-nitrosothiols. In parallel, nitrate fed mice had a reduction in body weight, visceral fat and circulating triglycerides whereas the control mice had unchanged body weight. Surprisingly, there were no differences in food intake between groups indicating that the weight loss in the nitrate group was explained by increased activity or changes in metabolic rate compared to the control group.

Figure 2

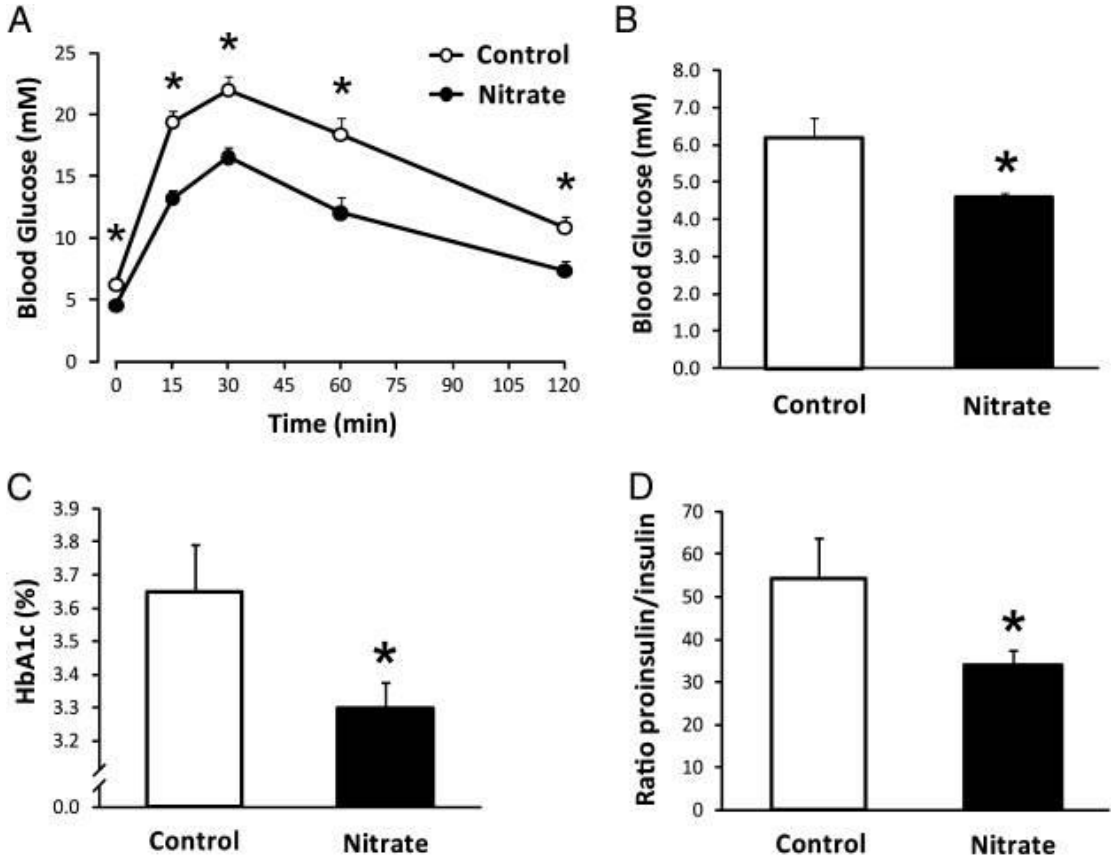

Figure 2) Dietary nitrate improves glucose tolerance and reduces fasting blood glucose in eNOS-deficient mice. (A) Effects on glucose tolerance after 10 weeks of dietary sodium nitrate supplementation (0.1 mmol $kg^{-1}d^{-1}$) following i.p. injection of glucose (2 g kg^{-1}) in controls (n = 11) and nitrate treated (n = 13) mice. (B) Effects on fasting glucose. (C) Effects on glycosylated hemoglobin (HbA1c) in nitrate treated (n = 8) and control mice (n = 10). (D) Effects on proinsulin-insulin ratios. Data are from the same animals as in B, but plasma was collected at the termination of the experiment after fasting for 14 h. Values are mean ± S.E.M.

Effects of inorganic nitrate on metabolism during exercise

Considering the known role of NO in regulation of energetics we wanted to explore the effects of dietary nitrate during controlled physical exercise. It was plausible that nitrate supplementation fuelling the nitrate-nitrite-NO pathway, could have metabolic and circulatory effects.

In study II oxygen consumption at all submaximal cycle ergometer workloads (45-85% of VO_2max) was reduced by on average 160 ml min^{-1} (p=0.02) after nitrate supplementation. VO_2max was not significantly different between conditions (4.61 ± 0.28 L min^{-1} after placebo and 4.49 ± 0.44 after nitrate, p = 0.29). However, maximal exercise testing during cycle ergometer exercise can be difficult in some individuals since VO_2max often is higher during uphill running or combined arm and leg exercise [109]. In study III we therefore investigated the effect of nitrate on VO_2max during maximal combined arm and leg exercise. During this type of exercise we found that VO_2max was reduced from 3.72 ± 0.33 to 3.62 ± 0.31 L min^{-1} in the placebo and nitrate conditions respectively (p=<0.05). Very surprisingly, the reduction in VO_2max was accompanied by a trend towards an increase in time to exhaustion (524 ± 31 vs 563 ± 30 seconds, in placebo and nitrate respectively, p=0.13). In this study we could also show that acute administration of nitrate affected oxygen consumption during submaximal exercise. In study V we used a single low-intensity workload and again we found that oxygen consumption was reduced from 1.95 ± 0.09 L min^{-1} during the placebo trial to 1.89 ± 0.1 L min^{-1} after nitrate supplementation (p=0.02). In all studies heart rate and pulmonary ventilation was unaffected by dietary nitrate. In study II and III RER was unchanged but in study V we found a slight increase in RER from 0.883 ± 0.01 in the placebo trial to 0.914 ± 0.01 in the nitrate trial, (p=0.02). This increase in RER indicating more carbohydrate oxidation could not fully explain the reduction in VO_2. In study II and III we measured capillary lactate concentrations before, during and after exercise at various workloads but found no significant differences.

Effects of dietary nitrate on basal mitochondrial function

With more than 90% of total oxygen consumption being of mitochondrial origin [98] we hypothesized that the reduction in oxygen consumption was due to mitochondrial alterations after nitrate ingestion. In study V we obtained muscle biopsies from *Vastus Lateralis* from healthy volunteers after three days of nitrate or placebo supplementation as previously described. Mitochondria were isolated and a variety of respiratory parameters were studies. Mitochondrial state 3 respiration with pyruvate and malate as substrates was unaffected by nitrate supplementation (see Figure 3). Likewise, when respiration was uncoupled by the ionophore FCCP, respiration increased compared to state 3 but were not different between conditions. However state 4 respiration, both in the presence and absence of atractyloside, was significantly lower after nitrate supplementation. Also LEAK-respiration, which is defined as the respiratory rate in the presence of substrates but without ADP, was significantly lower after nitrate. In study V we found q-values in the placebo condition of 0.966 that increased after nitrate supplementation to 0.985 ($p=0.01$, see Figure 3). As a consequence of the lower state 4 respiration, RCR values increased from 6.5 ± 0.7 to 8.5 ± 0.7 after nitrate, ($p=0.006$).

Figure 3

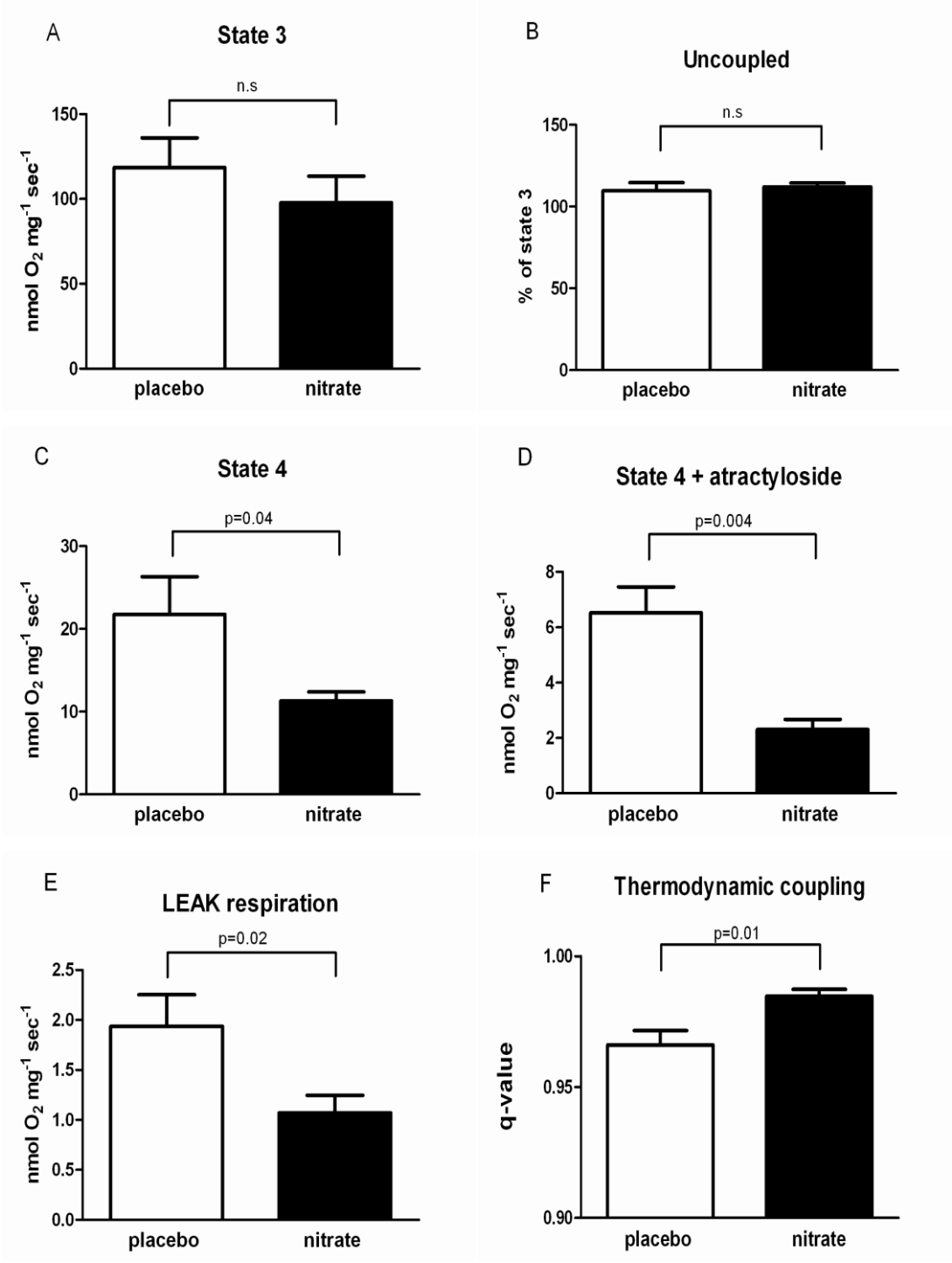

Figure 3) After three days of nitrate supplementation or placebo, skeletal muscle mitochondrial function was analyzed. (A) LEAK respiration (n = 9), (B) state 4 respiration (n = 14), and (C) state 4 respiration with atractyloside (n = 6) were lower after dietary nitrate compared to with placebo. (D) Nitrate supplementation did not influence oxygen consumption during state 3 respiration (n = 14) or (E) uncoupled respiration achieved by optimal titration with FCCP (n = 6). (F) Thermodynamic coupling (q value), was improved by dietary nitrate compared to placebo (n = 6). Data are mean ± S.E.M.

Effects of dietary nitrate on mitochondrial P/O ratio and oxygen affinity

A common criticism against studies with isolated mitochondria is that they do not reflect the true physiological state *in vivo*. Indeed, mitochondrial assays most often contain saturating concentrations of substrates and ADP and are performed under air saturated oxygen levels [58]. In two sets of experiments we tried to better mimic the physiological conditions in muscle tissue. In both these experiments pH was set to 6.7, resembling the intracellular pH during exercise [110]. We first measured the P/O ratio at non-saturating ADP concentrations similar to those during exercise [111]. After three days of nitrate supplementation the P/O ratio was improved from 1.36 ± 0.06 in the placebo trials to 1.62 ± 0.07 (p=0.02, see Figure 4a) after nitrate administration, indicating an improved mitochondrial efficiency. This increase in mitochondrial P/O ratio was strongly associated with the reduction in whole-body oxygen consumption in the same individuals, see Figure 4b.

Figure 4

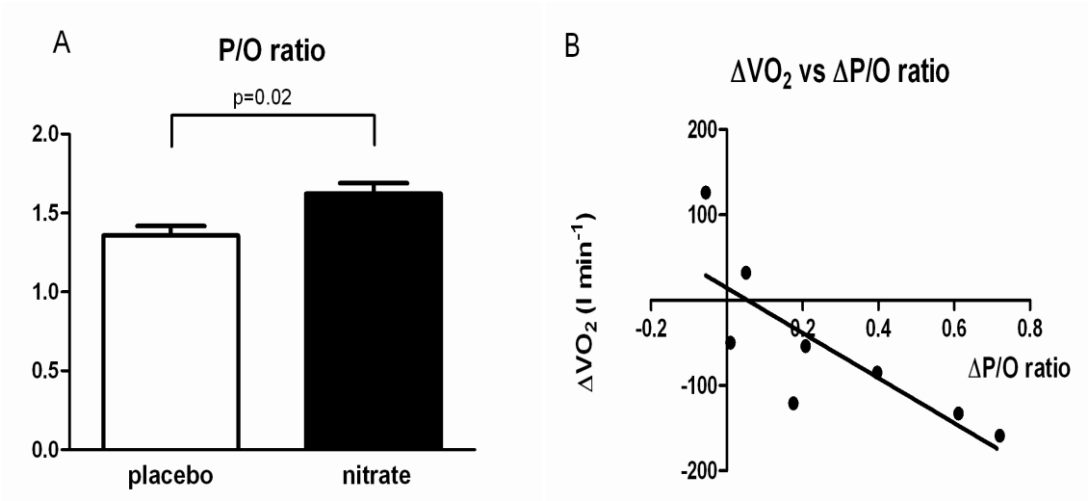

Figure 4) (A) Mitochondrial P/O ratio measured under steady-state infusion of non-saturating ADP-levels was improved after three days of nitrate supplementation. Values are mean ± S.E.M. (B) The increase in mitochondrial P/O ratio was significantly correlated with the decrease in whole-body oxygen consumption (R^2=0.64, p=0.02).

To study mitochondrial respiration at low oxygen tension, we measured the $p50_{mito}$ at state 3 respiration in response to nitrate supplementation. In the placebo condition $p50_{mito}$ was

0.042 ± 0.002 kPa and was near significantly increased to 0.053 ± 0.002 kPa (p=0.09). Interestingly, when nitrite was added *in vitro,* $p50_{mito}$ increased in a dose-dependent manner (see Figure 5).

Figure 5

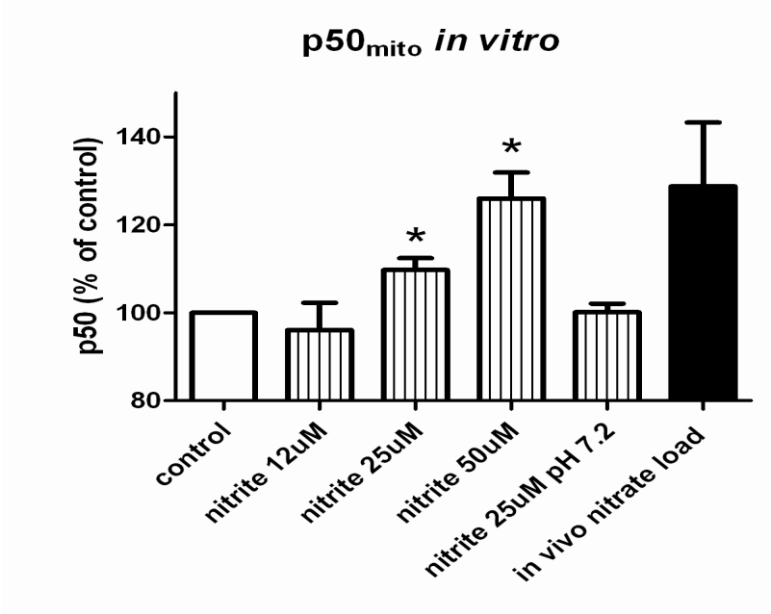

Figure 5) Mitochondrial oxygen affinity ($p50_{mito}$) was measured in skeletal muscle mitochondria after placebo treatment. When nitrite was added *in vitro,* $p50_{mito}$ increased in a dose dependent manner. When pH in the respiration medium was increased from 6.7 to 7.2 the effect was abolished. The effect of adding nitrite in vitro was compared to the effect after nitrate supplementation in vivo (black bar) that just failed to reach statistical significance (p=0.09). Values are mean ± S.E.M.

Effects of dietary nitrate on expression of putative uncoupling proteins

The reduced oxygen cost during exercise and the increased mitochondrial P/O ratio indicate that oxidative phosphorylation uncoupling is reduced after nitrate supplementation. Thus, we investigated the protein expression of two mitochondrial proteins, ANT and UCP-3, which have been proposed to uncouple respiration by allowing direct leakage of protons or by other means consuming the membrane potential [95, 112]. In study V we investigated the mitochondrial expression of these proteins by Western blotting and found that ANT was significantly reduced after nitrate supplementation (p=0.009). The reduction in UCP-3 expression failed to reach statistical significance (p=0.17) due to one outlier (see Figure 6).

Figure 6

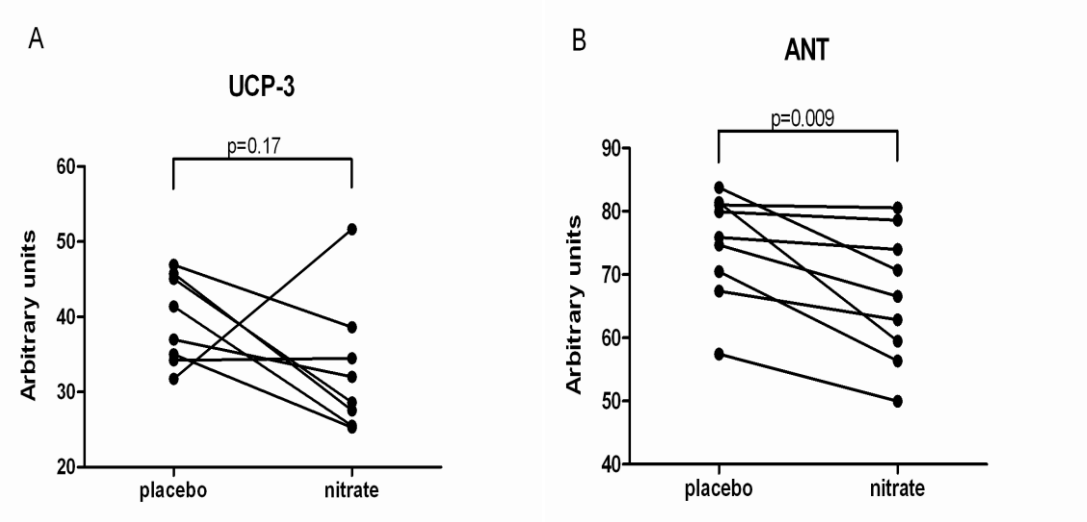

Figure 6) Total protein expression of (A) UCP-3 and (B) ANT, measured with Western blotting, after three days of placebo and nitrate supplementation.

Effect of dietary nitrate on mitochondrial biogenesis

In study V a series of experiments were performed to examine if the decreased expression of ANT and possibly UCP-3 represented a selective down regulation of these proteins or if overall mitochondrial biogenesis and density were lower after nitrate. First, we measured the activity of CS and COX since they are often used as markers of mitochondrial density. There was no evidence of a change in the activity of these two enzymes after nitrate supplementation (see Figure 7). The amount of mitochondrial DNA and nuclear DNA in the tissue samples were quantified using rt-PCR. The ratio (mtDNA/nDNA) is considered a hallmark of mitochondrial density. As can be seen in Figure 7, there was no difference in the mtDNA/nDNA ratio. Furthermore, the mRNA levels of three genes involved in mitochondrial biogenesis and function were investigated; peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1a), Transciption factor Alpha, mitochondrial (TFAM) and COX. Again, no differences were found in the gene expression between any of these genes (see Figure 7).

Figure 7

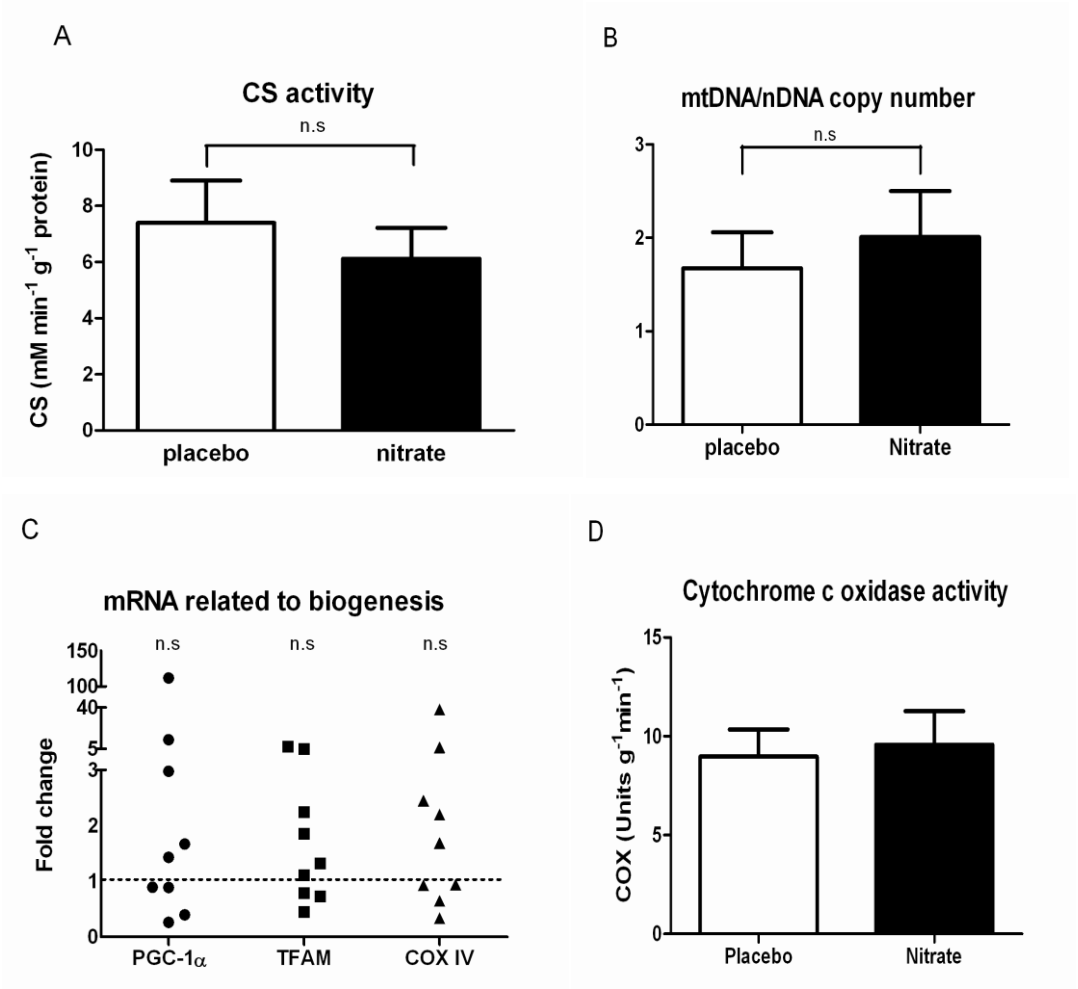

Figure 7) Dietary nitrate does not induce changes in mitochondrial density or biogenesis. (A) Citrate synthase activity, (B) mtDNA/nDNA ratio, (C) mRNA-levels of PGC-1α, TFAM and COX IV and (D) Cytochrome c oxidase activity were not affected by three days of supplementation with dietary nitrate. Values are mean ± S.E.M.

Mitochondrial oxygen affinity predicts basal metabolic rate

As explained above, metabolic efficiency or the efficiency of energy conversion is largely dictated by peripheral factors, the VO_2max on the other hand, is mainly limited by the maximal oxygen delivery capacity, which in turn is dictated by the pumping capacity of the heart [113]. Interestingly, VO_2max is readily increased by endurance training whereas efficiency during cycle ergometer exercise seems to be unaffected by such interventions [52, 114]. The finding that dietary nitrate seem to reduce both submaximal and maximal oxygen consumption indicates that there are more than one mechanism involved or that there is a link between metabolic efficiency and VO_2max. In study VI it was investigated if there were any qualitative mitochondrial differences between subjects with widely different BMR and metabolic efficiency. A strong correlation between BMR and the subjects'

$p50_{mito}$ (R^2=0.66, p=0.0004, see Figure 8) was found. A somewhat weaker relationship was found between cycling efficiency and $p50_{mito}$ (R^2=0.46, p=0.007). This indicates that there is a tight association between metabolic efficiency and the mitochondrial affinity for oxygen which theoretically could have an impact on the mitochondrial ability to extract oxygen when the oxygen availability is limiting such as during exercise [115].

Figure 8

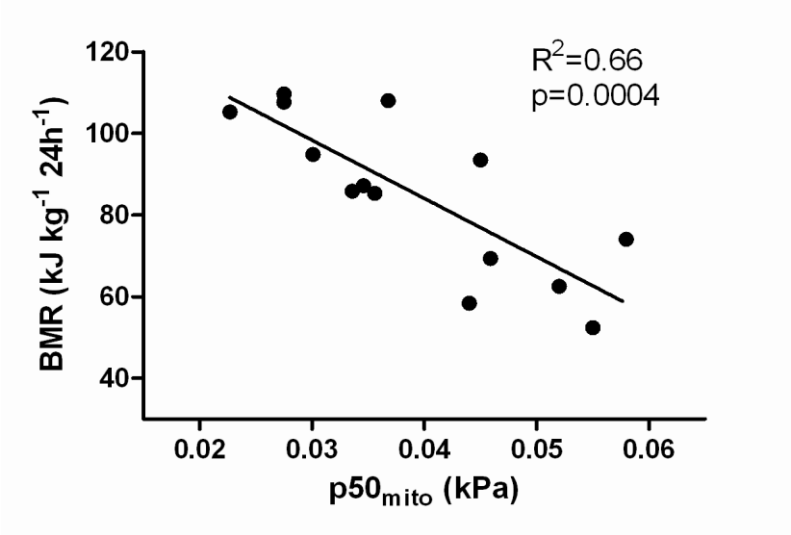

Figure 8) Association between basal metabolic rate, expressed per kg body mass, and the oxygen tension where mitochondrial respiration rate is half maximal ($p50_{mito}$).

GENERAL DISCUSSION

When the first studies in this thesis were initiated, the knowledge about the metabolic and circulatory effects of dietary nitrate was very limited. In 1994 two independent groups had shown that salivary nitrate, after reduction to nitrite by bacteria in the oral cavity, could generate NO in the human acidic stomach [21-22]. A role for gastric NO in host defence and gastric mucosal homeostasis was suggested. The year after Zweier and colleagues found endogenous NO generation from nitrite in the ischemic heart but related this production to myocardial injury and loss of contractile function [116]. In 2001 it was demonstrated that physiological amounts of nitrite could vasodilate aortic strips *in vitro*, an effect that was potentiated as pH in the medium decreased to levels seen in hypoxic or ischemic tissues. Vasodilation was paralleled by NO generation and abolished in the presence of a sGC-inhibitor, indicating that NO was the active substance [117]. In 2003 Gladwins' group went on and found that nitrite at near-physiological doses vasodilated the human circulation [30]. Then in 2004 Webb and colleagues demonstrated NO-dependent cardioprotective effects of nitrite in ischemia-reperfusion injury [118], a finding that now has been confirmed in a large number of studies, for review see [119]. In spite of these findings NO researchers still considered nitrate to be metabolically inert and it was actually used as a supposedly ineffective control substance in many experiments. In 2004 Lundberg and Govoni showed that ingestion of dietary nitrate increased plasma levels of nitrite, a pathway dependent on nitrate reducing bacteria in the oral cavity [19]. With these studies as a background, it was hypothesized that intake of dietary nitrate would fuel a nitrate-nitrite-NO pathway, generating NO and other bioactive nitrogen intermediates that could exert important biological effects.

Dietary nitrate and blood pressure

The results in study I, II and partly in III show that ingestion of dietary nitrate decreases blood pressure, an effect likely mediated by formation of NO. Blood pressure was also reduced after chronic dietary nitrate supplementation in rats in paper IV. The effect on blood pressure in humans has later been confirmed both with nitrate salts and beetroot juice as a natural source of nitrate [23, 108, 120-124]. Together these studies show both acute (single dose) and chronic blood pressure lowering effects. The latter suggests that there is no development of tolerance during nitrate intake in contrast to what is found with organic

nitrates [125]. Interestingly, Webb *et al.* and Petersson *et al.* found that when the enterosalivary circulation of nitrate and reduction to nitrite in the oral cavity was disrupted, either by spitting or the use of an antibacterial mouthwash, both the increase in plasma nitrite and decrease in blood pressure were abolished [23, 124]. This clearly shows that the reduction of nitrate to nitrite by oral commensal bacteria is a crucial first step in the bioconversion of nitrate to NO. The exact mechanism behind the blood pressure lowering effect is not fully elucidated. No nitrate-induced increase in plasma cGMP could be detected to support NO-mediated vasodilation but this has been described by Kapil *et al.* after ingestion of beetroot juice [108]. Although the most probable cause is vasodilation, cardiac and renal effects cannot be completely ruled out at this stage. Nitrite has been shown to have a negative inotropic effect in animal *ex vivo* studies [126].

Since vegetables contain high amounts of inorganic nitrate our findings clearly need to be put in a nutritional perspective. As of today nitrate is considered a potentially harmful and therefore undesired constituent in our diet and drinking water, in need of strict regulation. One the other hand there is consensus that a diet rich in vegetables promotes health. In fact such diets have been used in a controlled fashion to lower blood pressure (Dietary Approaches to Stop Hypertension, DASH) [51]. In these studies the blood pressure lowering effect was very similar to what was observed in our studies with inorganic nitrate. Other studies aiming at pinpointing the active components underlying the salutary effects of vegetables have not been successful. In a recent study Lansley and co-workers used a nitrate-depleted beetroot juice as placebo and found that this juice lacked the blood pressure lowering effects found with the natural juice [127]. Together, these data suggest the intriguing possibility that inorganic nitrate is an active ingredient in vegetables and may underlie their beneficial cardiovascular effects. Other salubrious effects of nitrate such as inhibition of platelet aggregation, improvement in endothelial function and protection against ischemia-reperfusion injury further support its role as a potentially beneficial component of a "green diet" [23].

Dietary nitrate and exercise

In the nitrate-nitrite-NO pathway the reduction of nitrite to NO and other nitrogen oxides is markedly enhanced during hypoxic and acidic conditions. In addition, NO is known to be involved in vasoregulation and modulation of cellular respiration, two important parts of the acute adaptation to exercise. Since both intracellular oxygen tension and pH is low in the working muscle during exercise it was plausible that dietary nitrate would have effects. Using human subjects and modern exercise physiology laboratory equipment, biopsies

were obtained in a cross-over fashion and steady-state metabolism was studied during highly standardized conditions, using methods with known good reproducibility.

The results from study II, III and V demonstrate that oxygen consumption is decreased after nitrate supplementation both at submaximal and maximal workloads. This is achieved without increases in plasma lactate indicating improved aerobic efficiency. In study V it was found that the reduction in oxygen consumption was coupled to an increased mitochondrial efficiency with improved P/O ratio, lower LEAK-respiration, lower state 4 respiration and a reduction in protein expression of ANT and possibly UCP-3. The increased P/O ratio correlated with the reduction in oxygen cost during exercise which strongly suggests that the observed improvement in mitochondrial efficiency is related to the oxygen sparing effects during exercise. The reduced oxygen consumption after nitrate supplementation seems to be a consistent finding during submaximal exercise [120-122, 128-131]. At maximal exercise the reduction is slightly more inconsistent with some studies showing decreased [121, 132-133] but others unchanged VO_2max [130-131]. The discrepancy between these results is not clear but the mode of exercise, dose and timing of nitrate supplementation and subject characteristics may all contribute to the divergent results. The exact mechanism how nitrate can reduce VO_2max is still unclear. The Fick equation states that:

$VO_2max = Q (CaO2-CvO2)$

where Q denotes cardiac output, CaO_2 is the oxygen content in arterial blood and CvO_2 is the oxygen content in venous blood. There are no indications of changes in Q has after nitrate supplementation, since maximal heart rate is unaffected. Further, the a-vO_2 difference is lower during exercise after nitrate supplementation [120]. This implies that the effect of nitrate lies primarily in the periphery with no substantial effect on cardiac output.

Does dietary nitrate improve physical performance?

An intriguing observation is that although several studies now show a decreased VO_2max by dietary nitrate, performance seems to improve [90-92, 97, 101] (see Table 4 for more details about performance during exercise). This is highly surprising since other known interventions that decrease VO_2max also decrease work performance accordingly. Examples of such interventions are hypoxic exposure [134], treatment with β-adreno receptor antagonists [135] and induced anemia [136]. The underlying mechanism for these two apparently paradoxical effects is unknown. In study VI a novel hypothesis is presented where $p50_{mito}$ is the common characteristic that unifies metabolic efficiency at rest and during submaximal exercise with VO_2max.

An increased efficiency during submaximal work should theoretically be beneficial for endurance performance since the same amount of work can be accomplished using less energy. Indeed, Horowitz *et al.* found that performance was different between two groups of cyclists with matched VO_2max but with significantly different gross efficiencies (20.4 vs 21.9%). During a one hour simulated time-trial the most efficient group of cyclists had an almost 10% higher average power output than the less efficient group [137]. In study II gross efficiency was increased from 19.7 to 21.1% after nitrate supplementation which theoretically could indicate an improved performance level. All studies summarized in Table 4 use time to exhaustion either at a fixed workload or during incremental exercise as measure of performance. A recent study by Lansley *et al* [127] instead used a time-trial design where competitive cyclists were instructed to cover a set distance at the fastest possible time, similar to a "real" athletic event. After nitrate supplementation, time to finish the distance was significantly reduced from 27.7 minutes to 26.9 minutes, indicating a substantially improved performance capacity. Future studies will reveal if dietary nitrate affect also other factors related to physical performance such as stimulation of the central nervous system, fatigue mechanisms, intracellular calcium levels etc.

Study	Subjects	Mode of nitrate administration	Work mode	Work time (min) nitrate	Work time (min) placebo	Time difference	Statistics
[131]	Young, healthy	BR, 6 days	Cycling	11:15	9:43	15.8%	p<0.05
[133]	Young, healthy	Sodium nitrate, 2 days	Arm and leg cycl.	9:23	8:44	7.5%	p=0.13
[132]	Cyclists, triathletes	Sodium nitrate, acute dose	Cycling	6:56	6:49	1.7%	p=0.17
[120]	PAD	BR, acute dose	Walking	8:53	7:47	17%	p<0.05
[129]	Young, healthy	BR, 6 days	Knee-extension	12:14	9:46	25%	p<0.01
[121]	Young, healthy	BR, 6 days	Running	8:42	7:36	15%	p<0.01

Table 4) Effects of acute or chronic supplementation with nitrate either as a sodium nitrate or with nitrate rich beetroot juice (BR) on time to exhaustion during exercise.

NO formation from dietary nitrate.

Although NO gas is readily detectable *in vitro* by laboratory techniques such as chemiluminescence, its detection *in vivo* is by no means straightforward. Instead of measuring NO directly, a variety of biomarkers or bioassays can be used as evidence of NO like activity, including cGMP-formation and citrulline to arginine ratio. In addition, measurable NO-like effects include proliferation of endothelial cells [138], stabilization of HIF-1 [139], the inhibition of COX [4] induction of nitrosative stress. The strongest evidence of NO-formation in the present thesis is the reduction in blood pressure that indicates peripheral vasodilation and the reduced oxygen consumption during exercise that might be interpreted as COX inhibition of NO or its chronic manifestations [140]. In addition, the fact that nitrate could partly reverse metabolic disturbances associated with the lack of eNOS, also indicates formation of NO or NO-like bioactivity.

An hypothesis how COX redox state controls metabolic efficiency, initiates cell signaling events and is shifted by dietary nitrate

As a last section in this thesis I would like to speculate on the temporal events and signaling pathways involved in the regulation of metabolism following nitrate intake. There seems to be both acute [127, 132-133] and chronic [120-121, 130-131, 141] effects on metabolism following nitrate administration. The acute effect, occurring within 1-3 hours after nitrate intake, could be related to nitrate being reduced to nitrite that is taken up by the tissue [142]. When the intracellular milieu is appropriate, as during exercise, nitrite is reduced to NO which subsequently inhibits COX. When NO binds to COX the enzyme is more reduced and slippage in the proton and/or electron transfer is attenuated [143], thereby improving the oxidative phosphorylation coupling and the whole-body efficiency, especially under conditions of limited oxygen availability. Simultaneously, the lower turnover of COX in the presence of NO increases the $p50_{mito}$ and thereby reduces the mitochondrial capacity to bind to and extract oxygen under normal physiological conditions. During severe intensity exercise the increased $p50_{mito}$ reduces VO_2max even though the oxygen transport from the heart is unaltered. However, the gain in efficiency is larger than the decrement in VO_2max so work performance is maintained or even increased. A potential outcome of sustained COX-inhibition by NO and a reduced ETS is that generation of radical species can be increased [84, 144]. Reaction products of superoxide, in particular H_2O_2 and $ONOO^-$, can initiate important signaling events involved in the cellular stress response. Such reactions might possibly explain the nitrate induced

structural modifications of proteins and down regulation of uncoupling proteins such as ANT and UCP-3 (study V) and irreversible inhibition of COX [140].

An interesting observation is that the physiological and mitochondrial adaptations after nitrate supplementation are similar to those expected after acute or chronic hypoxic exposure. For instance, although controversial, several studies show reduced oxygen consumption at a fixed workload after weeks of hypoxic exposure when measured at sea level [145-148]. Further, populations adapted for several generations to altitude seem to be metabolically more efficient, which is true both for Andeans [149] and for Tibetans [150]. Interestingly, another study shows that Tibetans have better metabolic efficiency and much higher exercise performance but lower VO_2max than a matched population residing at lower altitudes [151]. This could be indicative of chronic exposure to elevated NO-levels and a high $p50_{mito}$ as explained above. Indeed, a recent study also found that Tibetans have more than ten-fold higher circulating levels of nitrate and nitrite compared to sea-level residents, likely reflecting an upregulation of NOS activity [152].

To summarize, in this thesis we present novel biological functions of the mammalian nitrate-nitrite-NO pathway. By fuelling this pathway with dietary intake of inorganic nitrate we can achieve effects on blood pressure, mitochondrial function and oxygen utilization during exercise. In addition, nitrate supplementation reverses features of the metabolic syndrome in eNOS deficient mice. However, the true physiological role of the nitrate-nitrite-NO pathway in relation to the canonical NO-synthases still remains to be elucidated. Nevertheless, these findings implicate a therapeutic potential of nitrate as well as a nutritional role of this anion.

CONCLUSIONS

- Dietary nitrate has a mild blood pressure lowering effect when administered to humans and rodents.
- Many features of the metabolic syndrome in eNOS-deficient mice are attenuated after long-term treatment with nitrate in the drinking water.
- Whole-body oxygen uptake during submaximal as well as maximal exercise is reduced after nitrate administration. This occurs with unchanged or even improved physical performance.
- Mitochondrial efficiency improves after nitrate supplementation; this effect is directly correlated with the reduction in whole-body oxygen consumption.
- The improvement in mitochondrial efficiency is paralleled by a reduced expression of ANT, an important mitochondrial protein that has the potential to uncouple mitochondrial respiration.
- The $p50_{mito}$ can have important regulatory effects and is correlated to metabolic efficiency.
- Long term effects of nitrate administration has not been investigated but the traditional view that nitrate intake should be reduced to the lowest possible level is now seriously challenged.
- The benefits of short-term nitrate administration indicate that the relationship between nitrate-reducing bacteria in the oral cavity and the host mammal should be revised from “commensal” where only the bacteria benefits from the relationship and the host is unharmed, to “symbiotic” since both bacteria and the host seem to benefit from their relationship.

ACKNOWLEDGEMENTS

First of all I would like to express my gratitude towards all my supervisors for trusting me and giving me a huge amount of freedom during my PhD-education. It has been four rewarding years filled with many memorable and exiting moments.

Jon Lundberg, my main supervisor during my time as a PhD-student. Thanks for all the text-messages on late Saturday nights with your latest ideas on the next nitrate project. Your addiction to science seems to be contagious.

Eddie Weitzberg, my co-supervisor. Thanks for making the lab a happy place. Thanks for all good advice not only in science but also in life. Thanks for trying not to fall asleep in the calorimeter.

Björn Ekblom, my co-supervisor. Today I am really happy I sent you that e-mail four and a half years ago. It is truly inspiring to see someone like you that have been doing research for more than 45 years still being excited over our the latest data from the lab.

Tomas Schiffer, my closest colleague during the last two years. I appreciate your deep knowledge in organic chemistry but you really need to practice your drawing skills when trying to explain all those electron-transfer reactions to me. Thanks for all the laughs.

To all **human volunteers**, I am impressed by your engagement and willingness to endure the arduous exercise trials, donate muscle tissue and follow the strict dietary guidelines in my studies. Your contribution is important and much appreciated.

To the **mice and rats** that were sacrificed in study IV and VI. Well, it is too late to thank you now; all I can do is to hope that those studies will make a difference.

Thanks to **Ray, Efva, Sigrid, Simon, Jamal, Paulin and Johan** for offering me a place to sleep before I found an apartment when moving to Stockholm.

All **training buddies**, thanks for clearing my mind with gut-wrenching training sessions before work.

Thanks to all present and past co-workers and friends at the lab: **Mikael Mattson** for always having the answer, **William Apro** for your sincere kindness when helping out with the western blotting, **Örjan and Maria Ekblom** for support with statistics, **Frida Björkman** for making everyone else look like early-birds, **Michail Tonkonogi** for teaching me the basics in mitochondrial experimentation, **Niklas Psilander** for interesting discussions, **Kent Sahlin** for encouraging me to do the mitochondrial experiments, **Jonas Enqvist** for showing me were to buy the best coffee in Stockholm, **Mikael Flockhart** for all your n=1 experiments, **Jörgen Tannerstedt** for helping out in the lab**, Charlotte Schönbeck** for wearing high heels in the lab, **Marjan Pontén** for organizing all the get-

togethers, **Henrik Mascher** for your tantrums, **Elin Ekblom Bak** for your never-ending energy, **Marcus Borgenvik** for carrying heavy stuff up the stairs, **Per Berthelson** for coping with the old Clark-electrode, **Li Wang** for all the Chinese sweets, **Karin Söderlund** for always caring, **Peter Schantz** for encouraging me to bike to work every day, **Erik Stigell** and **Jane Salier** for being FAAPs, **Eva Blomqvist** for taking care of William, **Cecilia Jädert** for always smiling, **Meta Stensdotter** for treating the animals as good as possible, **Michael Hezel** for bringing cookies to the lab, **Raul Bescos** for your "pure mind", **Barbara Rocha** for solving all the problems**, Carina Nihlén** for your laughs, **Sara Borniquel** for teaching me useful phrases in Spanish, **Hiroaki Kishikawa** for being the most polite guy in the lab**, Liyue Huang** for being laid-back, **Annika Olsson** for making sure nobody gets lost when we are abroad.

Last but not least, I want to thank my little wonderful family, **Liza** and **Love**, for being the most valuable persons in my life and supporting me no matter what.

REFERENCES

1. Furchgott, R.F. and J.V. Zawadzki, *The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine.* Nature, 1980. **288**(5789): p. 373-6.
2. Ignarro, L.J., et al., *Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9265-9.
3. Rees, D.D., R.M. Palmer, and S. Moncada, *Role of endothelium-derived nitric oxide in the regulation of blood pressure.* Proc Natl Acad Sci U S A, 1989. **86**(9): p. 3375-8.
4. Brown, G.C. and C.E. Cooper, *Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase.* FEBS Lett, 1994. **356**(2-3): p. 295-8.
5. Cleeter, M.W., et al., *Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases.* FEBS Lett, 1994. **345**(1): p. 50-4.
6. Bolanos, J.P., et al., *Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes.* J Neurochem, 1994. **63**(3): p. 910-6.
7. Shen, W., T.H. Hintze, and M.S. Wolin, *Nitric oxide. An important signaling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption.* Circulation, 1995. **92**(12): p. 3505-12.
8. Allen, B.W., J.S. Stamler, and C.A. Piantadosi, *Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation.* Trends Mol Med, 2009. **15**(10): p. 452-60.
9. Lundberg, J.O., et al., *Nitrate, bacteria and human health.* Nat Rev Microbiol, 2004. **2**(7): p. 593-602.
10. Butler, A.R. and M. Feelisch, *Therapeutic uses of inorganic nitrite and nitrate: from the past to the future.* Circulation, 2008. **117**(16): p. 2151-9.
11. Rapoport, R.M., et al., *Effects of glyceryl trinitrate on endothelium-dependent and -independent relaxation and cyclic GMP levels in rat aorta and human coronary artery.* J Cardiovasc Pharmacol, 1987. **10**(1): p. 82-9.
12. Lindwall, R., et al., *A pilot study of inhaled nitric oxide in preterm infants treated with nasal continuous positive airway pressure for respiratory distress syndrome.* Intensive Care Med, 2005. **31**(7): p. 959-64.
13. Alving, K., E. Weitzberg, and J.M. Lundberg, *Increased amount of nitric oxide in exhaled air of asthmatics.* Eur Respir J, 1993. **6**(9): p. 1368-70.
14. Stuehr, D.J., et al., *Update on mechanism and catalytic regulation in the NO synthases.* J Biol Chem, 2004. **279**(35): p. 36167-70.
15. Richardson, R.S., et al., *Cellular PO2 as a determinant of maximal mitochondrial O(2) consumption in trained human skeletal muscle.* J Appl Physiol, 1999. **87**(1): p. 325-31.
16. Buerk, D.G., *Nitric oxide regulation of microvascular oxygen.* Antioxid Redox Signal, 2007. **9**(7): p. 829-43.
17. Thomas, D.D., et al., *The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2.* Proc Natl Acad Sci U S A, 2001. **98**(1): p. 355-60.
18. Shiva, S., et al., *Nitroxia: the pathological consequence of dysfunction in the nitric oxide-cytochrome c oxidase signaling pathway.* Free Radic Biol Med, 2005. **38**(3): p. 297-306.
19. Lundberg, J.O. and M. Govoni, *Inorganic nitrate is a possible source for systemic generation of nitric oxide.* Free Radic Biol Med, 2004. **37**(3): p. 395-400.
20. Duncan, C., et al., *Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate.* Nat Med, 1995. **1**(6): p. 546-51.

21. Lundberg, J.O., et al., *Intragastric nitric oxide production in humans: measurements in expelled air.* Gut, 1994. **35**(11): p. 1543-6.
22. Benjamin, N., et al., *Stomach NO synthesis.* Nature, 1994. **368**(6471): p. 502.
23. Webb, A.J., et al., *Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite.* Hypertension, 2008. **51**(3): p. 784-90.
24. Shiva, S., et al., *Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration.* Circ Res, 2007. **100**(5): p. 654-61.
25. Petersen, M.G., S. Dewilde, and A. Fago, *Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions.* J Inorg Biochem, 2008. **102**(9): p. 1777-82.
26. Castello, P.R., et al., *Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes.* Cell Metab, 2006. **3**(4): p. 277-87.
27. Webb, A.J., et al., *Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase.* Circ Res, 2008. **103**(9): p. 957-64.
28. Li, H., et al., *Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase.* J Biol Chem, 2008. **283**(26): p. 17855-63.
29. Reutov, V.P. and E.G. Sorokina, *NO-synthase and nitrite-reductase components of nitric oxide cycle.* Biochemistry (Mosc), 1998. **63**(7): p. 874-84.
30. Cosby, K., et al., *Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation.* Nat Med, 2003. **9**(12): p. 1498-505.
31. Feelisch, M., et al., *Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems.* J Biol Chem, 2008. **283**(49): p. 33927-34.
32. Hord, N.G., Y. Tang, and N.S. Bryan, *Food sources of nitrates and nitrites: the physiologic context for potential health benefits.* Am J Clin Nutr, 2009. **90**(1): p. 1-10.
33. Kilbourn, R.G., et al., *NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide.* Proc Natl Acad Sci U S A, 1990. **87**(9): p. 3629-32.
34. Tricker, A.R. and R. Preussmann, *Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential.* Mutat Res, 1991. **259**(3-4): p. 277-89.
35. van Loon, A.J., et al., *Intake of nitrate and nitrite and the risk of gastric cancer: a prospective cohort study.* Br J Cancer, 1998. **78**(1): p. 129-35.
36. McKnight, G.M., et al., *Dietary nitrate in man: friend or foe?* Br J Nutr, 1999. **81**(5): p. 349-58.
37. van Maanen, J.M., et al., *Consumption of drinking water with high nitrate levels causes hypertrophy of the thyroid.* Toxicol Lett, 1994. **72**(1-3): p. 365-74.
38. Ward, M.H., et al., *Nitrate intake and the risk of thyroid cancer and thyroid disease.* Epidemiology, 2010. **21**(3): p. 389-95.
39. Tang, L., et al., *Cruciferous vegetable intake is inversely associated with lung cancer risk among smokers: a case-control study.* BMC Cancer, 2010. **10**: p. 162.
40. Boffetta, P., et al., *Fruit and vegetable intake and overall cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC).* J Natl Cancer Inst, 2010. **102**(8): p. 529-37.
41. Freedman, N.D., et al., *Fruit and vegetable intake and head and neck cancer risk in a large United States prospective cohort study.* Int J Cancer, 2008. **122**(10): p. 2330-6.
42. Ford, E.S., W.H. Giles, and W.H. Dietz, *Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey.* JAMA, 2002. **287**(3): p. 356-9.
43. Monti, L.D., et al., *Endothelial nitric oxide synthase polymorphisms are associated with type 2 diabetes and the insulin resistance syndrome.* Diabetes, 2003. **52**(5): p. 1270-5.

44. Fernandez, M.L., et al., *Association of NOS3 gene with metabolic syndrome in hypertensive patients.* Thromb Haemost, 2004. **92**(2): p. 413-8.
45. Duplain, H., et al., *Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase.* Circulation, 2001. **104**(3): p. 342-5.
46. Merry, T.L., G.S. Lynch, and G.K. McConell, *Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction.* Am J Physiol Regul Integr Comp Physiol, 2010. **299**(6): p. R1656-65.
47. Henstridge, D.C., et al., *The effect of the nitric oxide donor sodium nitroprusside on glucose uptake in human primary skeletal muscle cells.* Nitric Oxide, 2009. **21**(2): p. 126-31.
48. Smukler, S.R., et al., *Exogenous nitric oxide and endogenous glucose-stimulated beta-cell nitric oxide augment insulin release.* Diabetes, 2002. **51**(12): p. 3450-60.
49. Nisoli, E., et al., *Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide.* Science, 2003. **299**(5608): p. 896-9.
50. Wang, C.H., C.C. Wang, and Y.H. Wei, *Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes.* Ann N Y Acad Sci, 2010. **1201**: p. 157-65.
51. Appel, L.J., et al., *A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group.* N Engl J Med, 1997. **336**(16): p. 1117-24.
52. Moseley, L., et al., *No differences in cycling efficiency between world-class and recreational cyclists.* Int J Sports Med, 2004. **25**(5): p. 374-9.
53. Åstrand PO, R.K., Dahl HA, Strömme SB, *Textbook of Work Physiology. Physiological bases of Exercise.* 4th ed. 2003.
54. Gaesser, G.A. and G.A. Brooks, *Muscular efficiency during steady-rate exercise: effects of speed and work rate.* J Appl Physiol, 1975. **38**(6): p. 1132-9.
55. Mogensen, M., et al., *Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency.* J Physiol, 2006. **571**(Pt 3): p. 669-81.
56. Nickleberry, B.L., Jr. and G.A. Brooks, *No effect of cycling experience on leg cycle ergometer efficiency.* Med Sci Sports Exerc, 1996. **28**(11): p. 1396-401.
57. Boning, D., Y. Gonen, and N. Maassen, *Relationship between work load, pedal frequency, and physical fitness.* Int J Sports Med, 1984. **5**(2): p. 92-7.
58. Gnaiger, E., G. Mendez, and S.C. Hand, *High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia.* Proc Natl Acad Sci U S A, 2000. **97**(20): p. 11080-5.
59. Befroy, D.E., et al., *Increased substrate oxidation and mitochondrial uncoupling in skeletal muscle of endurance-trained individuals.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16701-6.
60. Ortenblad, N., W.A. Macdonald, and K. Sahlin, *Glycolysis in contracting rat skeletal muscle is controlled by factors related to energy state.* Biochem J, 2009. **420**(2): p. 161-8.
61. Bassett, D.R., Jr. and E.T. Howley, *Limiting factors for maximum oxygen uptake and determinants of endurance performance.* Med Sci Sports Exerc, 2000. **32**(1): p. 70-84.
62. Celsing, F., et al., *Effects of anaemia and stepwise-induced polycythaemia on maximal aerobic power in individuals with high and low haemoglobin concentrations.* Acta Physiol Scand, 1987. **129**(1): p. 47-54.
63. Bergh, U., I.L. Kanstrup, and B. Ekblom, *Maximal oxygen uptake during exercise with various combinations of arm and leg work.* J Appl Physiol, 1976. **41**(2): p. 191-6.
64. Astrand, P.O. and B. Saltin, *Maximal oxygen uptake and heart rate in various types of muscular activity.* J Appl Physiol, 1961. **16**: p. 977-81.
65. Gleser, M.A., D.H. Horstman, and R.P. Mello, *The effect on Vo2 max of adding arm work to maximal leg work.* Med Sci Sports, 1974. **6**(2): p. 104-7.
66. Secher, N.H., et al., *Maximal oxygen uptake during arm cranking and combined arm plus leg exercise.* J Appl Physiol, 1974. **36**(5): p. 515-8.

67. Holmberg, H.C., H. Rosdahl, and J. Svedenhag, *Lung function, arterial saturation and oxygen uptake in elite cross country skiers: influence of exercise mode.* Scand J Med Sci Sports, 2007. **17**(4): p. 437-44.
68. Boushel, R., et al., *Muscle mitochondrial capacity exceeds maximal oxygen delivery in humans.* Mitochondrion, 2011. **11**(2): p. 303-7.
69. Mole, P.A., et al., *Myoglobin desaturation with exercise intensity in human gastrocnemius muscle.* Am J Physiol, 1999. **277**(1 Pt 2): p. R173-80.
70. de Groot, H., T. Noll, and H. Sies, *Oxygen dependence and subcellular partitioning of hepatic menadione-mediated oxygen uptake. Studies with isolated hepatocytes, mitochondria, and microsomes from rat liver in an oxystat system.* Arch Biochem Biophys, 1985. **243**(2): p. 556-62.
71. Bakkman, L., et al., *Quantitative and qualitative adaptation of human skeletal muscle mitochondria to hypoxic compared with normoxic training at the same relative work rate.* Acta Physiol (Oxf), 2007. **190**(3): p. 243-51.
72. Gnaiger, E., et al., *Mitochondrial respiration in the low oxygen environment of the cell. Effect of ADP on oxygen kinetics.* Biochim Biophys Acta, 1998. **1365**(1-2): p. 249-54.
73. Boveris, A., et al., *Regulation of mitochondrial respiration by oxygen and nitric oxide.* Ann N Y Acad Sci, 2000. **899**: p. 121-35.
74. Koivisto, A., et al., *Kinetics of the inhibition of mitochondrial respiration by NO.* FEBS Lett, 1997. **417**(1): p. 75-80.
75. Shen, W., et al., *Role of nitric oxide in the regulation of oxygen consumption in conscious dogs.* Circ Res, 1994. **75**(6): p. 1086-95.
76. Ishibashi, Y., et al., *ATP-sensitive K+ channels, adenosine, and nitric oxide-mediated mechanisms account for coronary vasodilation during exercise.* Circ Res, 1998. **82**(3): p. 346-59.
77. Lacerda, A.C., et al., *Evidence that brain nitric oxide inhibition increases metabolic cost of exercise, reducing running performance in rats.* Neurosci Lett, 2006. **393**(2-3): p. 260-3.
78. Heinonen, I.H., et al., *Skeletal muscle blood flow and oxygen uptake at rest and during exercise in humans: a PET study with nitric oxide and cyclooxygenase inhibition.* Am J Physiol Heart Circ Physiol, 2011.
79. Loke, K.E., et al., *Nitric oxide modulates mitochondrial respiration in failing human heart.* Circulation, 1999. **100**(12): p. 1291-7.
80. Recchia, F.A., et al., *Nitric oxide controls cardiac substrate utilization in the conscious dog.* Cardiovasc Res, 1999. **44**(2): p. 325-32.
81. Nunez, C., et al., *Discrepancies between nitroglycerin and NO-releasing drugs on mitochondrial oxygen consumption, vasoactivity, and the release of NO.* Circ Res, 2005. **97**(10): p. 1063-9.
82. Antunes, F., A. Boveris, and E. Cadenas, *On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide.* Proc Natl Acad Sci U S A, 2004. **101**(48): p. 16774-9.
83. Gnaiger, E., et al., *Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase.* J Exp Biol, 1998. **201**(Pt 8): p. 1129-39.
84. Poderoso, J.J., et al., *Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles.* Arch Biochem Biophys, 1996. **328**(1): p. 85-92.
85. Rossignol, R., et al., *Mitochondrial threshold effects.* Biochem J, 2003. **370**(Pt 3): p. 751-62.
86. Thomas, D.D., et al., *Hypoxic inducible factor 1alpha, extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of nitric oxide.* Proc Natl Acad Sci U S A, 2004. **101**(24): p. 8894-9.
87. Wikstrom, M.S., M., *Bioenergetics,* ed. L. Ernester. 1984, Amsterdam: Elsevier.
88. Clerc, P., et al., *Nitric oxide increases oxidative phosphorylation efficiency.* J Bioenerg Biomembr, 2007. **39**(2): p. 158-66.
89. Murphy, M.P. and M.D. Brand, *Variable stoichiometry of proton pumping by the mitochondrial respiratory chain.* Nature, 1987. **329**(6135): p. 170-2.

90. Capitanio, N., et al., *H+/e- stoichiometry of mitochondrial cytochrome complexes reconstituted in liposomes. Rate-dependent changes of the stoichiometry in the cytochrome c oxidase vesicles.* FEBS Lett, 1991. **288**(1-2): p. 179-82.
91. Kadenbach, B., et al., *Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase.* Free Radic Biol Med, 2000. **29**(3-4): p. 211-21.
92. Shen, W., et al., *Endogenous nitric oxide enhances coupling between O2 consumption and ATP synthesis in guinea pig hearts.* Am J Physiol Heart Circ Physiol, 2001. **281**(2): p. H838-46.
93. Gnaiger, E., *Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply.* Respir Physiol, 2001. **128**(3): p. 277-97.
94. Conley, K.E., et al., *Mitochondrial function, fibre types and ageing: new insights from human muscle in vivo.* Exp Physiol, 2007. **92**(2): p. 333-9.
95. Bevilacqua, L., et al., *Absence of uncoupling protein-3 leads to greater activation of an adenine nucleotide translocase-mediated proton conductance in skeletal muscle mitochondria from calorie restricted mice.* Biochim Biophys Acta, 2010. **1797**(8): p. 1389-97.
96. Brand, M.D., et al., *The basal proton conductance of mitochondria depends on adenine nucleotide translocase content.* Biochem J, 2005. **392**(Pt 2): p. 353-62.
97. Hoeks, J., M.K. Hesselink, and P. Schrauwen, *Involvement of UCP3 in mild uncoupling and lipotoxicity.* Exp Gerontol, 2006. **41**(7): p. 658-62.
98. Rolfe, D.F. and M.D. Brand, *Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate.* Am J Physiol, 1996. **271**(4 Pt 1): p. C1380-9.
99. Virtanen, K.A., et al., *Functional brown adipose tissue in healthy adults.* N Engl J Med, 2009. **360**(15): p. 1518-25.
100. Zingaretti, M.C., et al., *The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue.* FASEB J, 2009. **23**(9): p. 3113-20.
101. Kadenbach, B., *Intrinsic and extrinsic uncoupling of oxidative phosphorylation.* Biochim Biophys Acta, 2003. **1604**(2): p. 77-94.
102. Carlstrom, M., et al., *Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice.* Proc Natl Acad Sci U S A, 2010. **107**(41): p. 17716-20.
103. Brouwer, E., *On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (Oxygen intake and carbonic acid output) and urine-N.* Acta Physiol Pharmacol Neerl, 1957. **6**: p. 795-802.
104. Steinlechner-Maran, R., et al., *Oxygen dependence of respiration in coupled and uncoupled endothelial cells.* Am J Physiol, 1996. **271**(6 Pt 1): p. C2053-61.
105. Gnaiger, E., et al., *Control of mitochondrial and cellular respiration by oxygen.* J Bioenerg Biomembr, 1995. **27**(6): p. 583-96.
106. Cairns, C.B., et al., *Mitochondrial oxidative phosphorylation thermodynamic efficiencies reflect physiological organ roles.* Am J Physiol, 1998. **274**(5 Pt 2): p. R1376-83.
107. Reisch, A.S. and O. Elpeleg, *Biochemical assays for mitochondrial activity: assays of TCA cycle enzymes and PDHc.* Methods Cell Biol, 2007. **80**: p. 199-222.
108. Kapil, V., et al., *Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO.* Hypertension, 2010. **56**(2): p. 274-81.
109. Hermansen, L., B. Ekblom, and B. Saltin, *Cardiac output during submaximal and maximal treadmill and bicycle exercise.* J Appl Physiol, 1970. **29**(1): p. 82-6.
110. Sahlin, K., et al., *Intracellular pH and bicarbonate concentration in human muscle during recovery from exercise.* J Appl Physiol, 1978. **45**(3): p. 474-80.
111. Willis, W.T. and M.R. Jackman, *Mitochondrial function during heavy exercise.* Med Sci Sports Exerc, 1994. **26**(11): p. 1347-53.

112. Parker, N., et al., *Energization-dependent endogenous activation of proton conductance in skeletal muscle mitochondria.* Biochem J, 2008. **412**(1): p. 131-9.
113. Andersen, P. and B. Saltin, *Maximal perfusion of skeletal muscle in man.* J Physiol, 1985. **366**: p. 233-49.
114. Marsh, A.P., P.E. Martin, and K.O. Foley, *Effect of cadence, cycling experience, and aerobic power on delta efficiency during cycling.* Med Sci Sports Exerc, 2000. **32**(9): p. 1630-4.
115. Richardson, R.S., et al., *Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability.* J Physiol, 2006. **571**(Pt 2): p. 415-24.
116. Zweier, J.L., P. Wang, and P. Kuppusamy, *Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy.* J Biol Chem, 1995. **270**(1): p. 304-7.
117. Modin, A., et al., *Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation.* Acta Physiol Scand, 2001. **171**(1): p. 9-16.
118. Webb, A., et al., *Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13683-8.
119. Dezfulian, C., et al., *Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics.* Cardiovasc Res, 2007. **75**(2): p. 327-38.
120. Kenjale, A.A., et al., *Dietary Nitrate Supplementation Enhances Exercise Performance in Peripheral Arterial Disease.* J Appl Physiol, 2011.
121. Lansley, K.E., et al., *Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study.* J Appl Physiol, 2011. **110**(3): p. 591-600.
122. Vanhatalo, A., et al., *Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise.* Am J Physiol Regul Integr Comp Physiol, 2010. **299**(4): p. R1121-31.
123. Sobko, T., et al., *Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers.* Nitric Oxide, 2010. **22**(2): p. 136-40.
124. Petersson, J., et al., *Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash.* Free Radic Biol Med, 2009. **46**(8): p. 1068-75.
125. Dejam, A., et al., *Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation.* Circulation, 2007. **116**(16): p. 1821-31.
126. Pellegrino, D., et al., *Nitrite exerts potent negative inotropy in the isolated heart via eNOS-independent nitric oxide generation and cGMP-PKG pathway activation.* Biochim Biophys Acta, 2009. **1787**(7): p. 818-27.
127. Lansley, K.E., et al., *Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance.* Med Sci Sports Exerc, 2011.
128. Larsen, F.J., et al., *Dietary inorganic nitrate improves mitochondrial efficiency in humans.* Cell Metab, 2011. **13**(2): p. 149-59.
129. Bailey, S.J., et al., *Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans.* J Appl Physiol, 2010. **109**(1): p. 135-48.
130. Larsen, F.J., et al., *Effects of dietary nitrate on oxygen cost during exercise.* Acta Physiol (Oxf), 2007. **191**(1): p. 59-66.
131. Bailey, S.J., et al., *Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans.* J Appl Physiol, 2009. **107**(4): p. 1144-55.
132. Bescos, R., et al., *Acute Administration of Inorganic Nitrate Reduces VO2peakin EnduranceAthletes.* Med Sci Sports Exerc, 2011.
133. Larsen, F.J., et al., *Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise.* Free Radic Biol Med, 2010. **48**(2): p. 342-7.
134. Peltonen, J.E., et al., *Effects of oxygen fraction in inspired air on rowing performance.* Med Sci Sports Exerc, 1995. **27**(4): p. 573-9.

135. Jilka, S.M., et al., *Maximal exercise responses to acute and chronic beta-adrenergic blockade in healthy male subjects.* Med Sci Sports Exerc, 1988. **20**(6): p. 570-3.
136. Celsing, F., et al., *Effect of long-term anemia and retransfusion on central circulation during exercise.* J Appl Physiol, 1986. **61**(4): p. 1358-62.
137. Horowitz, J.F., L.S. Sidossis, and E.F. Coyle, *High efficiency of type I muscle fibers improves performance.* Int J Sports Med, 1994. **15**(3): p. 152-7.
138. Isenberg, J.S., et al., *Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner.* Proc Natl Acad Sci U S A, 2005. **102**(37): p. 13141-6.
139. Li, F., et al., *Regulation of HIF-1alpha stability through S-nitrosylation.* Mol Cell, 2007. **26**(1): p. 63-74.
140. Cooper, C.E., et al., *Nitric oxide and peroxynitrite cause irreversible increases in the K(m) for oxygen of mitochondrial cytochrome oxidase: in vitro and in vivo studies.* Biochim Biophys Acta, 2003. **1607**(1): p. 27-34.
141. Bailey, S.J., et al., *Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans.* J Appl Physiol, 2010.
142. Bryan, N.S., et al., *Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury.* Proc Natl Acad Sci U S A, 2007. **104**(48): p. 19144-9.
143. Muramoto, K., et al., *Bovine cytochrome c oxidase structures enable O2 reduction with minimization of reactive oxygens and provide a proton-pumping gate.* Proc Natl Acad Sci U S A, 2010. **107**(17): p. 7740-5.
144. Poyton, R.O., K.A. Ball, and P.R. Castello, *Mitochondrial generation of free radicals and hypoxic signaling.* Trends Endocrinol Metab, 2009. **20**(7): p. 332-40.
145. Green, H.J., et al., *Increases in submaximal cycling efficiency mediated by altitude acclimatization.* J Appl Physiol, 2000. **89**(3): p. 1189-97.
146. Gore, C.J., et al., *Live high:train low increases muscle buffer capacity and submaximal cycling efficiency.* Acta Physiol Scand, 2001. **173**(3): p. 275-86.
147. Katayama, K., et al., *Intermittent hypoxia improves endurance performance and submaximal exercise efficiency.* High Alt Med Biol, 2003. **4**(3): p. 291-304.
148. Saunders, P.U., et al., *Improved running economy in elite runners after 20 days of simulated moderate-altitude exposure.* J Appl Physiol, 2004. **96**(3): p. 931-7.
149. Hochachka, P.W., et al., *Metabolic and work efficiencies during exercise in Andean natives.* J Appl Physiol, 1991. **70**(4): p. 1720-30.
150. Marconi, C., et al., *Economy of locomotion in high-altitude Tibetan migrants exposed to normoxia.* J Physiol, 2005. **569**(Pt 2): p. 667-75.
151. Ge, R.L., et al., *Higher exercise performance and lower VO2max in Tibetan than Han residents at 4,700 m altitude.* J Appl Physiol, 1994. **77**(2): p. 684-91.
152. Erzurum, S.C., et al., *Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans.* Proc Natl Acad Sci U S A, 2007. **104**(45): p. 17593-8.