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

MITOCHONDRIAL EFFICIENCY

FOCUS ON DIETARY NITRATE, HYPOXIA AND EXERCISE

Tomas Schiffer

Karolinska
Institutet

Stockholm 2014

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AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen
försvaras i föreläsningssal Atrium (54), Karolinska Institutet Solna.

Fredagen den 14 november, 2014, kl 09.00

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ABSTRACT

Metabolic efficiency affects weight control, generation of heat, exercise performance and health. The tiny radical nitric oxide (NO) targets several cellular components that can influence metabolic efficiency. NO is produced endogenously from L-arginine and molecular oxygen by specific NO-synthases. In addition, extensive research during the past two decades shows that the inorganic anions nitrate and nitrite, which are oxidation products from endogenous NO generation, can be reduced back to NO and other nitrogen oxides. Apart from reflecting endogenous NOS-activity, circulating nitrate and nitrite are dependent on dietary intake, where green leafy vegetables in particular contain high amounts of nitrate. Circulating nitrate is actively taken up by the salivary glands and excreted in saliva. Oral commensal bacteria then reduce salivary nitrate into nitrite, which after swallowing and effective uptake in the gut, reaches the systemic circulation. In blood and tissues several enzymatic and non-enzymatic pathways are able to reduce nitrite to NO. These pathways are potentiated under acidic and hypoxic conditions. The nitrate-nitrite-NO pathway is considered a back-up system during conditions when NO-synthases are failing. Prior to the inception of this thesis, our group had shown that dietary nitrate was able to decrease oxygen cost during exercise and we wanted to further explore its metabolic effects

With this background, we investigated the underlying mechanisms behind the oxygen sparing effect of dietary nitrate during exercise **(Paper I)**. Moreover, we explored the effects of dietary nitrate on muscular function in mice **(Paper II)**, oxygen consumption in a human model of global hypoxia **(Paper III)** and on resting metabolic rate in humans **(Paper IV)**. In the last two papers we wanted to investigate if metabolic efficiency can affect exercise tolerance in hypoxia **(Paper V)** and how cytochrome c oxidase (COX) subunit IV isoform composition affects resting metabolic rate **(Paper VI)**.

Respirometric analysis of isolated mitochondria from healthy humans revealed that dietary nitrate improves mitochondrial efficiency (P/O ratio) and this effect correlated strongly with the reduction in oxygen consumption during cycling ergometry. In addition we found respirometric support for less uncoupling which was supported by reduced expression of adenine nucleotide transporter (ANT), a major determinant of proton conductance **(Paper I)**. In muscle from mice fed for 7 days with nitrate, electric stimulation led to increased contractile force and speed of force development in fast twitch muscle compared to controls. This was accompanied by higher Ca^{2+} levels and increased expression of the Ca^{2+}-handling proteins dihydropyridine receptor and calsequestrin-1 **(Paper II)**. In the human model of global hypoxia a reduction in arterial oxygen saturation was achieved during prolonged breath-holding by experienced free divers after nitrate or placebo. In contrast to our hypothesis, nitrate during resting apnea increased pulmonary oxygen uptake, reduced arterial oxygen saturation and shortened maximal breath-holding time. This was probably related to a NO-mediated attenuation of the oxygen conserving diving response as showed by less bradycardia and indications of an attenuation of the increase in blood pressure after nitrate **(Paper III)**. In healthy humans we could demonstrate that dietary nitrate reduces resting metabolic rate by 4% and that acute administration of nitrite *in vitro* reduces respiration by 40% in primary myotubes from the same individuals **(Paper IV)**. We found that healthy subjects with a high metabolic efficiency in normoxia had higher tolerance to exercise in hypoxia. Interestingly, these subjects acutely reduced their metabolic efficiency during hypoxia in order to maintain power output. On the other hand, isolated mitochondria, which work in the lower efficiency range, acutely increased their efficiency during a steady state hypoxic challenge in order to maintain ATP production **(Paper V)**. There is a largely unexplained variation in resting metabolic rate between seemingly similar individuals. We found that the inter-individual variation in resting metabolic rate seems to depend on the composition of COX subunit IV isoforms. We could show that COX IV-2 isoform is present in human skeletal muscle and that a high COX IV-2/COX IV-1 ratio showed a strong negative correlation to resting metabolic rate. Moreover, concurrent overexpression of COX IV-2 and knock down of COX IV-1 in primary human myotubes significantly reduced basal cell respiration and ROS generation without affecting the COX activity **(Paper VI)**.

In conclusion, this thesis demonstrates profound effects of dietary nitrate on mitochondrial efficiency, muscle function and metabolism. In addition, metabolic efficiency plays a role in exercise tolerance during hypoxia and adapts to obtain optimal power. Finally, mitochondrial COX subunit IV isoform composition seems to affect resting metabolic rate. The physiological, therapeutic and nutritional aspects of these findings create a platform for further studies on dietary nitrate, mitochondrial function and metabolism.

LIST OF PUBLICATIONS

I. **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.* *Equal contribution.*

II. **Dietary nitrate increases tetanic [Ca2+]i and contractile force in mouse fast-twitch muscle.**
Hernández A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E, Westerblad H.
J Physiol. 2012 Aug 1;590(Pt 15):3575-83

III. **Effects of dietary inorganic nitrate on static and dynamic breath-holding in humans.**
Schiffer TA, Larsen FJ, Lundberg JO, Weitzberg E, Lindholm P.
Respir Physiol Neurobiol. 2013 Jan 15;185(2):339-48.

IV. **Dietary nitrate reduces resting metabolic rate: a randomized, crossover study in humans.**
Larsen FJ, Schiffer TA, Ekblom B, Mattsson MP, Checa A, Wheelock CE, Nyström T, Lundberg JO, Weitzberg E.
Am J Clin Nutr. 2014 Apr;99(4):843-50.

V. **Dynamic regulation of metabolic efficiency explains tolerance to acute hypoxia in humans.**
Schiffer TA, Ekblom B, Lundberg JO, Weitzberg E, Larsen FJ.
FASEB J. 2014 Jun 26. pii: fj.14-251710.

VI. **Subunit composition of cytochrome c oxidase predicts resting metabolic rate.**
Schiffer TA, Larsen FJ, Ekblom B, Lundberg JO, Weitzberg E
Manuscript

RELATED PAPERS NOT INCLUDED IN THE THESIS

1. Mitochondrial oxygen affinity predicts basal metabolic rate in humans.
Larsen FJ, **Schiffer TA**, Sahlin K, Ekblom B, Weitzberg E, Lundberg JO.
FASEB J. 2011 Aug;25(8):2843-52.

2. Regulation of mitochondrial function and energetics by reactive nitrogen oxides.
Larsen FJ, **Schiffer TA**, Weitzberg E, Lundberg JO.
Free Radic Biol Med. 2012 Nov 15;53(10):1919-28.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
α-KGDH	α-ketoglutarate dehydrogenase
Akt	Protein kinase B
ANT	Adenine nucleotide transporter
ATP	Adenosine triphosphate
BMR	Basal metabolic rate
BSA	Bovine serum albumin
$(C_a - C_v)$	Arteriovenous oxygen difference
CASQ1	Calsequestrin-1
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cytochrome c oxidase
COX IV-1	Cytochrome c oxidase subunit IV isoform 1
COX IV-2	Cytochrome c oxidase subunit IV isoform 2
CR	Caloric restriction
CS	Citrate synthase
DNA	Deoxyribonucleic acid
DHPR	Dihydropyridine receptor
ΔpH	Proton gradient
$\Delta\Psi_m$	Transmembrane electrical potential
EDL	Extensor digitorum longus (muscle)
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ETS	Electron transport system
$FADH_2$	Flavin adenine dinucleotide (reduced)
FBS	Fetal bovine serum
$FETCO_2$	End tidal fraction of carbon dioxide
$FETO_2$	End tidal fraction of oxygen
FCCP	Carbonyl cyanide 3-chlorophenylhydrazone
FDB	Flexor digitorum brevis
FFM	Fat free mass
FiO_2	Fraction of inhaled oxygen

FO$_2$	Fraction of oxygen
GE	Gross efficiency
H$_2$O$_2$	Hydrogen peroxide
HPLC	High performance liquid chromatography
HRmax	Maximum heart rate
HRR	High resolution respirometry
iNOS	Inducible nitric oxide synthase
kPa	Kilopascal
LL	Large loss group
MAO	Monoamine oxidase
MAP	Mean arterial blood pressure
ME	Metabolic efficiency
mtDNA	Mitochondrial deoxyribonucleic acid
N$_2$	Nitrogen gas
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
nDNA	Nuclear deoxyribonucleic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX4	NADPH oxidase 4
NRF	Nuclear respiratory factor
OEMP	Optimal efficiency for maximum power
ONOO$^-$	Peroxynitrite
P50$_{mito}$	Oxygen tension were mitochondrial respiration is half-maximal
PCO$_2$	Partial pressure of carbon dioxide
PCr	Phosphocreatine
PDE2A	Phosphodiesterase 2A
PGC-1alpha	Peroxisome proliferator-activated receptor gamma
P$_i$	Inorganic phosphate
PKC	Protein kinase C
P/O ratio	Adenosine triphosphate/oxygen ratio
PO$_2$	Partial pressure of oxygen
PVDF	Polyvinylidene fluoride
q	Thermodynamic coupling
RCR	Respiratory control ratio

RER	Respiratory exchange ratio
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RyR1	Ryanodine receptor 1
sAC	Soluble adenylyl cyclase
SaO_2	Arterial oxygen saturation
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
sGC	Soluble guanylate cyclase
siRNA	Small interfering ribonucleic acid
SL	Small loss group
SNO	S-nitroso thiol
SR	Sarcoplasmic reticulum
T3	Triiodothyronine
T4	Thyroxine
TCA-cycle	Tricarboxylic acid cycle
TFAM	Transcription factor A, mitochondrial
TSH	Thyroid-stimulating hormone
UCP-3	Uncoupling protein 3
VCO_2	Volume of produced carbon dioxide
VEmax	Maximal ventilation
VO_2	Volume of consumed oxygen
VO_2MAX	Maximal oxygen consumption (large muscle groups)
VO_2peak	Maximal oxygen consumption (Smaller muscle group)
W_{max}	Exercise capacity
XOR	Xanthine oxidoreductase

INTRODUCTION

Nitrogen gas (N_2) is the most abundant element in the atmosphere and is essential for all living organisms. In order to be biologically useful, nitrogen needs to undergo fixation by prokaryotes (diazotrophs) whereby nitrogen is converted to ammonium (NH_4^+). Ammonium can then be oxidized to nitrite (NO_2^-), nitrate (NO_3^-) and a variety of other nitrogen oxides. Bacteria eventually reduce these higher nitrogen oxides back to nitrogen gas, which is released into the atmosphere and this denitrification process completes the nitrogen cycle in nature. Bacteria are involved in all steps of the nitrogen cycle and are therefore essential for life as nitrogen is a required component in proteins, DNA and RNA.

Analogous to the reduction of oxygen at cytochrome c oxidase, the terminal enzyme of the mitochondrial electron transport system, bacteria use nitrate and nitrite as terminal electron acceptors and for incorporation in biomass. The earliest life on earth emerged around 3.5 billion years ago (Schopf, 2006) and according to the endosymbiosis hypothesis, around 2 billion years later, the mitochondrion evolved from a bacterial progenitor via symbiosis within an essentially eucaryotic host cell (Gray, 2012) offering nutrients and shelter and in return benefited from an efficient energy generating system. This symbiosis has prevailed since then and we now know that mitochondria have numerous functions in the cell apart from solely generating ATP. In this thesis we have studied the role of a mammalian nitrogen cycle involving nitrate, nitrite and nitric oxide (NO) in mitochondrial function and metabolism.

Human metabolism

The basal metabolic rate (BMR) represents the minimum energy required to sustain life. It can span as much as 900-2200 kcal day^{-1} between different subjects and normally represents the largest component of the daily caloric expenditure. For example the net energy expenditure during a 10 km run by an average man is around 700 kcal, meaning that even in relatively active subjects the basal metabolic rate is still the dominant part of energy expenditure. The maintenance of cell membrane ionic gradients through the activity of Na-K ATPase and Ca^{2+}-ATPase consumes significant amounts of ATP and has been implicated in around 25% of the total oxygen consumption at rest (rewieved in Clausen et al., 1991). Biosynthesis, RNA/DNA turnover, signal transduction etc. also contribute to BMR (Clausen et al., 1991). In addition, thyroid hormones (Johnstone et al., 2005) and brown adipose tissue are known to play a role (Yoneshiro and Saito, 2014). Fat free mass (FFM) is the major factor determining BMR (Weinsier et al., 1992). Muller and colleagues found that oxygen consumption in muscle, kidney, brain and liver explains up to 43% of the inter-individual variance in resting metabolic rate (Müller et al., 2011). However, a large part of the intraspecific variation is still unexplained (Johnstone et al., 2005). Variations in mitochondrial uncoupling affecting the efficiency of oxidative phosphorylation may explain these variations and are discussed later.

Mitochondrial function

In 1961, Peter Mitchell proposed the chemiosmotic theory suggesting that the main part of ATP synthesis originates from energy stored by the electrochemical gradient across the inner mitochondrial membrane (Mitchell, 1961). In 1978, Mitchell was awarded the Nobel Prize in chemistry for this finding. The reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), formed in

the tricarboxylic acid (TCA) cycle, supply electrons to the electron transport system where electron transfer is driven by the redox potential of the individual components of the respiratory system (Fig. 1). A proton motive force is then generated through the drop in redox potential during the transfer of electrons. Electrons are ultimately transferred to molecular oxygen at cytochrome c oxidase (COX) via four-electron transfers. The transmembrane electrochemical gradient is formed via the pumping of protons from the mitochondrial matrix across the inner membrane and is facilitated by the three complexes: NADH oxidase, cytochrome bc_1 complex and COX. The proton motive force then drives the flow of protons back to the matrix through ATP synthase that combines adenosine diphosphate (ADP) and inorganic phosphate to adenosine triphosphate (ATP) (Fig. 1).
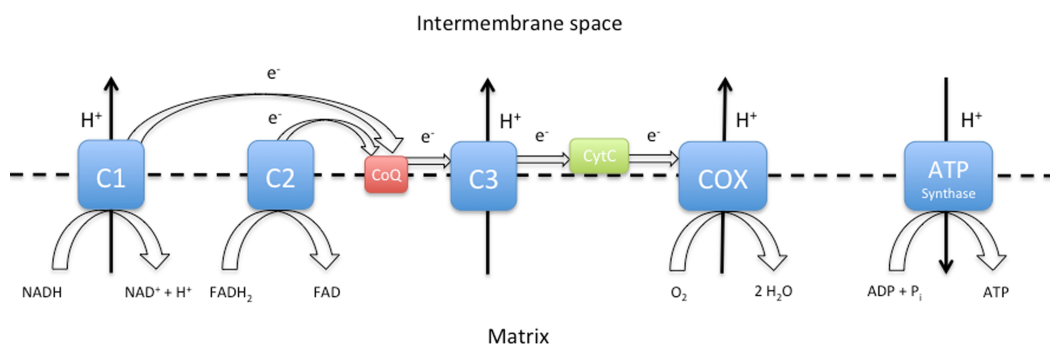

Figure 1. Schematic view of the electron transport system.

Uncoupling of oxidative phosphorylation

Not all mitochondrial oxygen consumption is coupled to ATP synthesis. The uncoupling of oxidative phosphorylation in mammals is largely mediated by mitochondrial carrier proteins (Dulloo and Samec, 2001) of which the most prominent is uncoupling protein 1 (UCP-1), present in brown adipose tissue where it facilitates non-shivering thermogenesis (Cannon and Nedergaard, 2010). It has been estimated that up to 20% of BMR can be attributed to incomplete coupling of oxidative phosphorylation (Rolfe and Brown, 1997). Speakman and colleagues showed that higher levels of endogenous activators of proton conductance through adenine nucleotide translocase (ANT) and UCP-3 contributed to this proton leak (Speakman et al., 2004).

Regulation of cytochrome c oxidase activity

ATP/ADP ratio

At high cellular energy levels, ATP functions as an allosteric inhibitor of COX, adjusting energy production to cellular demand. COX contains several ADP binding sites of which the majority are exchangeable for ATP at high ATP/ADP ratios (Napiwotzki and Kadenbach, 1998; Napiwotzki et al., 1997). These multiple binding sites confer allosteric inhibition (Malatesta et al., 1987), modifications in cytochrome c affinity (Bisson et al., 1987) and changes in energy transduction efficiency (Kadenbach et al., 1998). The transmembrane COX subunit IV with its ATP/ADP binding sites is the key subunit responsible for regulation of COX activity (Arnold and Kadenbach, 1997). The allosteric inhibition is dependent on the presence of cardiolipin (Arnold and Kadenbach, 1997). In addition, COX subunit Va serves as a

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binding site for 3,5-diiodo-L-thyronine (T2) that results in reduction of ATP-mediated allosteric inhibition (Arnold et al., 1998). COX subunit IV exists in two isoforms; COX IV-1 is ubiquitously expressed in mammalian tissues while COX IV-2 has previously only been found in human adult lung, fetal lung and muscle (Hüttemann et al., 2001) and in neurons (Horvat et al., 2006; Misiak et al., 2010; Singh et al., 2010). COX IV-2 causes an abolishment of allosteric COX inhibition by ATP and is suggested to suppress COX sensitivity of energy detection and adjustments to cellular energy needs (Horvat et al., 2006; Misiak et al., 2010; Singh et al., 2010).

H^+/e^- stoichiometry

At high ATP/ADP ratios, a decrease in COX H^+/e^- stoichiometry (1.0 to 0.5) has been described (Frank and Kadenbach, 1996), leading to a reduction in COX efficiency of energy transduction at a low cellular workload. This mechanism has been suggested to contribute to the maintenance of resting body temperature. In addition, an increase in transmembrane electrical potential ($\Delta\Psi_m$) and proton gradients (ΔpH) decrease H^+/e^- stoichiometry, thereby diminishing efficiency of energy transduction (Murphy and Brand, 1987) (Papa et al., 1991). Subunit VIa also exhibits a decrease of H^+/e^- stoichiometry at low concentrations of palmitate, implying that fatty acids may affect the efficiency of energy production (Lee and Kadenbach, 2001). It is not known whether the exchange of the previously mentioned subunits COX IV-1 to COX IV-2 contributes to changes in H^+/e^- stoichiometry.

Post-translational modification through phosphorylation

Steenart & Shore were the first to demonstrate phosphorylation at COX subunit IV by an endogenous kinase (Steenaart and Shore, 1997). Later Bender & Kadenbach showed that incubation of COX and rat heart mitochondria with protein kinase A, cAMP and ATP resulted in serine and threonine phosphorylation of COX subunits I and II as well as subunit Vb which could be reversed by Ca^{2+}-activated dephosphorylation (Bender and Kadenbach, 2000). It was suggested that the phosphorylation of COX subunit I turned on allosteric ATP inhibition (Helling et al., 2008). On the other hand, activation of PKC leads to COX subunit IV phosphorylation (Ogbi et al., 2004) and increased activity (Ogbi and Johnson, 2006). In contrast, Avadhani and colleagues showed that ischemia reperfusion in Langendorff perfused rabbit hearts caused PKA-mediated phosphorylation of subunit I, IV and Vb that reduced COX activity and increased ROS production (Prabu et al., 2006). Acin-Perez and coworkers demonstrated that metabolically generated carbon dioxide promotes HCO_3^--dependent activation of mitochondrial soluble adenylyl cyclase (sAC). The subsequent production of cAMP activates mitochondrial PKA which in turn phosphorylates and activates COX (Acin-Perez et al., 2009). Later, the same group identified a mitochondrial matrix located phosphodiesterase (PDE2A) necessary for switching of the cAMP signal (Acin-Perez et al., 2011).

Mitochondria and generation of reactive oxygen species

Already in 1966 it was demonstrated that mitochondria produce reactive oxygen species (ROS) (Jensen, 1966). Since then the physiological relevance of data obtained using artificial systems such as isolated mitochondria has been debated. Although excessive ROS production is implicated in the pathogenesis of many diseases (Parkinson's disease, Alzheimer's disease, Huntington's disease) physiological ROS

generation has important roles in signal transduction (Dröge, 2002). ROS generation at complex III has been demonstrated, however only during artificial conditions in which this complex is inhibited by antimycin (Chen et al., 2003). Not even the loss of cytochrome c, resulting in the reduction of the entire respiratory system, leads to ROS formation at complex III (Turrens et al., 1985). The physiological relevance of ROS production at complex III has therefore been questioned. Instead complex I seems to be the main site of ROS generation although it is still debated which components of complex I are involved. The physiological relevance for complex I-mediated ROS formation in healthy mitochondria has also been questioned. Grivennkova & Vinogradov showed that NADH-supported superoxide production in submitochondrial particles was bell-shaped, showing maximal activity at unphysiologically low NADH concentrations while at physiological levels superoxide production was severely inhibited (Grivennikova and Vinogradov, 2006).

The consequence of COX inhibition is a reduction of the electron transport system that may enhance superoxide production. Accordingly, treatment of isolated mitochondria with NO, that reversibly binds to and inhibits COX activity, increases superoxide production (Poderoso et al., 1996). NO-mediated superoxide production can in turn modulate a variety of signal transduction cascades involved in adaptive responses and cellular defense mechanisms (Erusalimsky and Moncada, 2007). Apart from the components in the respiratory system, significant amount of ROS can be produced by monoamine oxidases (MAOs) located in the outer membrane of mitochondria (Andreyev et al., 2005), the TCA cycle enzyme α-ketoglutarate dehydrogenase (α-KGDH) (Tretter and Adam-Vizi, 2004) and NADPH oxidase 4 (NOX4) (Block et al., 2009).

Endogenous nitric oxide synthesis

Nitric oxide (NO) is a free radical and a key physiological messenger involved in a variety of important cellular functions such as vasodilation, platelet aggregation, neurotransmission and immune responses. This tiny uncharged gas molecule diffuses freely across membranes, making it an ideal autocrine and paracrine signalling molecule. NO is biosynthesized from L-arginine, oxygen, and nicotinamide adenine dinucleotide phosphate (NADPH) by NO synthases (NOSs) that exists in 3 isoforms; endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Moncada et al., 1997). The name reveals that eNOS was initially discovered in endothelial cells, where it is constitutively expressed and activated by fluid shear stress, intracellular Ca^{2+} increase or phosphorylation through Akt and 5' AMP-activated protein kinase (AMPK), which can be induced by estrogen or insulin (Forstermann and Sessa, 2012). nNOS is also constitutively expressed and is predominately found in neurons, heart/skeletal muscle and pancreatic islet cells (Daff, 2010; Murad, 2006). iNOS is mainly expressed in cells of the immune system and is induced by lipopolysaccharide and cytokines (Stuehr et al., 1991). Soluble guanulyl cyclase (sGC) is the classical NO target and activation of sGC by NO binding induces formation of the second messenger 3', 5'-cyclic guanosine monophosphate (cGMP) which in the vasculature ultimately results in relaxation of vascular smooth muscle cells. NO is short lived *in vivo* with a half life of a few seconds and is rapidly oxidized to nitrite and nitrate which are commonly used as markers of NOS activity (Lauer et al., 2001).
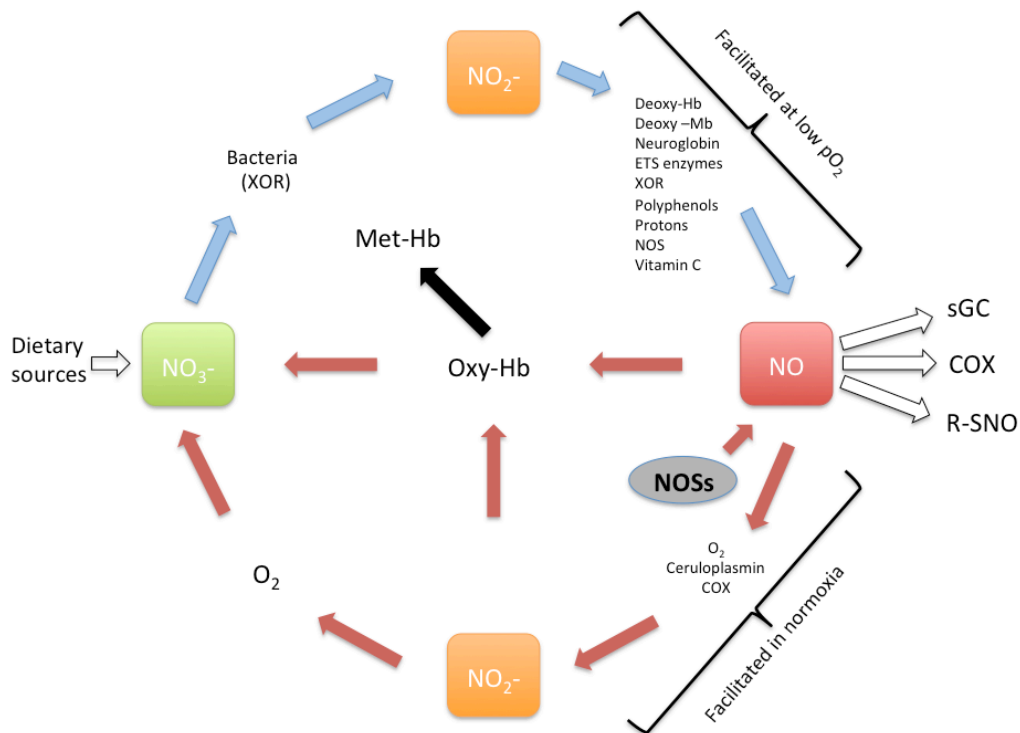
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Figure 2. Schematic view of the mammalian nitrate–nitrite–NO cycle. Dietary and endogenous sources of nitrate are reduced to nitrite by oral bacteria (and to lesser extent by xanthine oxidoreductase (XOR) in the tissues). At low oxygen tensions, reduction of nitrite to NO can be catalyzed by acidic reduction, antioxidants or several different enzymes and proteins. NO can then signal via the classical activation of soluble guanulyl cyclase (sGC), via formation of S-nitrosothiols (SNO) and cytochrome c oxidase (COX) with important regulatory functions. NO is enzymatically oxidized back to nitrite by ceruloplasmin and COX or by autooxidation. NO also reacts with haemoglobin which yields nitrate and methemoglobin (Met-Hb). Deoxy-Hb=deoxyhemoglobin, Deoxy-Mb=deoxymyoglobin, ETS enzymes=enzymes in the mitochondrial electron transport system and NOS=nitric oxide synthase.

The nitrate-nitrite-NO pathway

The inorganic anions nitrate and nitrite are oxidation products derived from endogenous NOS-dependent NO generation. These anions were earlier considered to be mostly inert until the mid 1990s when it was discovered that salivary nitrite can be non-enzymatically reduced back to NO in the acidic stomach (Benjamin et al., 1994; Lundberg et al., 1994). Later it was shown that nitrite can be reduced to NO in various tissues via several different enzymatic systems and that NO production through these pathways is potentiated during hypoxia and low pH (Lundberg and Weitzberg, 2010) (Fig. 2). In addition to the nitrate derived from endogenous NO synthesis, this anion is also supplied by our diet, with the highest levels found in green leafy vegetables such as ruccola, spinach and beetroots. After ingestion, more than 25% of circulating nitrate is actively taken up by the salivary glands and secreted into the oral cavity (Qin et al., 2012). Oral commensal bacteria then reduce parts of the salivary nitrate to nitrite (Spiegelhalder et al., 1976) and following ingestion of nitrite-rich saliva and efficient absorption in the gastrointestinal tract, systemic nitrite reduction leads to generation of NO-like bioactivity (Lundberg et al., 2008; van Faassen et al., 2009; Weitzberg and Lundberg, 1998). As this pathway is mainly facilitated during hypoxia

and acidic conditions, it is suggested to serve as a back up system in hypoxia when NO synthesis by the oxygen-dependent NOSs is suppressed (Lundberg et al., 2009; van Faassen et al., 2009). The possibility to fuel this pathway by dietary nitrate in combination with the well established protective effects of a diet rich in vegetables on cardiovascular disease opened up a whole new research field investigating therapeutic and nutritional implications of the nitrate-nitrite-NO pathway. Several studies have now shown that dietary nitrate reduces blood pressure, inhibits platelet aggregation, protects against ischemia-reperfusion injury in several organ systems and reduces oxygen cost during exercise (Weitzberg and Lundberg, 2013).

Nitric oxide and mitochondrial function

NO has the ability to competitively (with oxygen) and reversibly bind to COX thereby inhibiting mitochondrial respiration. It has been calculated that half inhibition of COX is obtained at an O_2/NO ratio of around 500 (Boveris et al., 2000). In humans, COX possesses a pronounced overcapacity relative to the maximum flux of the electron transport system (Antunes et al., 2004; Gnaiger et al., 1998a) suggesting that severe inhibition of COX is required before it is possible to see an effect on respiration. A higher COX capacity relative to the upstream complexes positively affects the mitochondrial oxygen affinity (Gnaiger et al., 1998a). This overcapacity may be vital to prevent severe inhibition of COX by NO under normal physiological conditions (Gnaiger et al., 1998b). Still, systemic NO synthase inhibition in humans increases oxygen consumption at rest and during exercise (Heinonen et al., 2011) indicating that physiological NO levels are high enough to impact oxygen consumption (Thomas et al., 2004). At physiological oxygen tensions of around 1 kPa (10 μM), COX would already be half-inhibited by physiological NO levels and even further inhibited in working muscle where oxygen tension may decrease to as low as 0.03 kPa (Molé et al., 1999; Richardson et al., 2001). Another result of inhibiting COX that may have impact on oxygen consumption is improved efficiency of oxidative phosphorylation (Clerc et al., 2007). This is attributed to reduced proton slippage (Clerc et al., 2007) and is further supported by the observed increase in oxygen consumption during inhibition of endogenous NO production without changes in ATP production (Shen et al., 2001).

Nitrite and mitochondrial function

Several components of the respiratory system have the capacity to function as nitrite reductases (Basu et al., 2008; Castello et al., 2006; Kozlov et al., 1999). In 1999 Kozlov and colleagues demonstrated NADH-dependent nitrite reduction at complex III in anoxia in submitochondrial particles from bovine heart at a nitrite concentration of 50 μM (Kozlov et al., 1999). Later, Castello and coworkers found a pH-dependent nitrite reductase activity by COX at high nitrite concentrations (1mM) and this occurred only below a pO_2 of 2 kPa (Castello et al., 2006). In addition, Basu and colleagues showed that cytochrome c, in which heme iron normally is in six-coordinate state, can work as a nitrite reductase in penta-coordination (Basu et al., 2008). This nitrite reductase activity was facilitated in anoxic and acidic conditions.

Exercise physiology

Oxygen consumption (VO_2) is defined by the Fick principle: $VO_2 = CO \times (C_a - C_v)$ where CO is the cardiac output and ($C_a - C_v$) is the arteriovenous oxygen difference. In 1923 Hill and colleagues proposed that maximal cardiac output was the primary

factor explaining individual differences in maximal oxygen consumption (VO_2max) and it is still considered to be the main limiting factor during whole body exercise performance. The higher cardiac output seen in trained subjects reflects larger maximal stroke volume, given that considerably less variation exists in maximal heart rate and systemic oxygen extraction. The oxygen carrying capacity attributable by the total content of haemoglobin is another important variable that may alter VO_2max. For instance, blood doping through reinfusion of 900-1350 ml blood improves VO_2max by 4-9% (Gledhill, 1985). Training promotes an increased capillary density (Andersen and Henriksson, 1977) which correlates with VO_2max (Saltin et al., 1977). This enhances oxygen extraction even at high rates of muscle blood flow by elongating mean transit time (Saltin, 1985). In elite athletes, the pulmonary system may limit VO_2max by the reduced transit time of the red blood cells in the pulmonary capillary which may not be enough to saturate the blood with O_2 (Dempsey et al., 1984). In mixed venous blood draining maximally working muscle, oxygen saturation roughly falls to around 10 to 15% corresponding to an oxygen content of $\approx$ 30 ml O_2 L^{-1}. (Astrand et al., 1964). Thus, there is a potential for extracting more or less oxygen depending not only on capillarisation but also on qualitative properties of the mitochondria. The potential impact of qualitative properties of skeletal muscle mitochondria on arteriovenous oxygen difference at maximal exercise has been poorly investigated. However, during moderate hypoxia, the apparent Km for ADP in presence of creatine has been shown to positively correlate with the maximal a-v O_2 difference pointing towards a mitochondrial regulation in moderate hypoxia (Ponsot et al., 2010). In addition, the age related decline in VO_2max seems to be due primarily to impaired efficiency of maximal peripheral oxygen extraction (McGuire et al., 2001). The structural capacity of oxygen diffusion is unchanged with age where the capillary density in type I fibers is maintained (McGuire et al., 2001). Therefore, the reduction of mitochondrial oxidative capacity or oxygen affinity seems to play an important role in the decline of VO_2 max in scenescense. Cytochrome c oxidase activity seem to decline with age as well (Tonkonogi et al., 2003) which actually may have profound effects on the mitochondrial oxygen affinity (Gnaiger et al., 1998a) and in turn partly explain the reduced basal metabolic rate with ageing (Schrack et al., 2014). With improved techniques for measuring mitochondrial oxygen affinity it is now possible to investigate the relationship between this variable, VO_2max and arteriovenous oxygen difference and explore whether it is possible to change this variable by training or dietary regimens.

Muscle contraction

Muscle fibers are stimulated via the release of acetylcholine at the motor end plate leading to an end plate current that activates fast voltage-gate Na^+-channels. This initiates the firing of an action potential along the entire muscle fiber which penetrates the depths of the fiber via the transverse tubules (T-tubules). The depolarization of the fiber activates voltage-sensitive dihydropyridine receptors (DHPR) that are in close connection and communicates with the ryanodine receptors (RyR1) (Tanabe et al., 1990) located in the adjacent membrane of sarcoplasmic reticulum (SR). The depolarization leads to a conformational change of DHPR that causes the RyR1 to open and allows the flow of stored Ca^{2+} from the SR into the cytosol. Troponin-C binds Ca^{2+} and the inhibitory effect of tropomyocin is thereby cancelled and enables ATP-dependent filament sliding to occur by the high affinity actin-myocin II binding. The Ca^{2+} signal that leads to contraction of striated muscle is largely dependent on the Ca^{2+} storage capacity of SR. Calsequestrin-1 (CASQ1) is a protein with a high capacity and low affinity for Ca^{2+} ions and is therefore a very efficient Ca^{2+} storage protein in SR

and maintains free Ca^{2+} concentration at ~1mM (Beard et al., 2004). CASQ1 is shown to regulate SR Ca^{2+} and RyRs and therefore affects $[Ca^{2+}]_i$ and force during contractions (Aydin et al., 2009; Beard et al., 2004; Canato et al., 2010; Paolini et al., 2007). During muscle relaxation, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) transfers Ca^{2+} back from the cytosol to the SR lumen. Thus, changes in the expression of DHPR, RyR1, SERCA and CASQ1 have the potential to affect Ca^{2+}-handling and in turn the excitation-contraction coupling.

Dietary nitrate and exercise performance

In 2007, Larsen and colleagues showed that 3 days of sodium nitrate supplementation to healthy young subjects reduced oxygen cost during submaximal exercise (Larsen et al., 2007). The reduced oxygen cost was not associated with changes in heart rate, minute ventilation or elevated plasma [lactate], indicative of improvement in aerobic efficiency. This was intriguing since at the time, the oxygen cost at a given workload was considered to be a relatively fixed parameter and previously reported to be unaffected by chronic or acute interventions. Later Bailey and colleagues confirmed the reduction of oxygen consumption during exercise by a 3 day administration of nitrate-containing beetroot juice (Bailey et al., 2009). Then followed a series of corroborating studies showing reduced oxygen cost as a result of nitrate administration (Cermak et al., 2012; Lansley et al., 2011b; Larsen et al., 2010; Vanhatalo et al., 2010). These results implied that dietary nitrate had the potential to enhance exercise performance. Accordingly, Lansley and colleagues investigated whether acute dietary nitrate supplementation with beetroot juice affected power output and performance during cycling time trials. The intervention increased mean power output by 4.6% and improved performance by 2.8% (Lansley et al., 2011a). Later, the results were confirmed by a study where 6 days of beetroot juice improved time trial performance by 1.2% in trained cyclists (Cermak et al., 2012). However, Bescós and colleagues reported that after a 3 day period of sodium nitrate in trained athletes, no changes were observed in terms of mean power output or mean distance covered during a 40 min cycle ergometer distance trial test (Bescos et al., 2012). Similar results were obtained when sodium nitrate was acutely administered to highly trained cross-country skiers in a 5 km time trial (Peacock et al., 2012). The higher resting plasma concentration of nitrate and nitrite due to higher eNOS activity in extremely well trained individuals may explain the resistance toward nitrate-mediated improvements in exercise performance (Jungersten et al., 1997; Schena et al., 2002).

Bailey and colleagues used ^{31}P-magnetic resonance spectroscopy to investigate changes in contractile efficiency during knee-extensor exercise after nitrate administration (Bailey et al., 2010). 6 days of beetroot juice supplementation reduced ATP turnover rates from phosphocreatine (PCr) hydrolysis and oxidative phosphorylation. It also blunted intramuscular accumulation of ADP and inorganic phosphate (Pi) as well as the extent of PCr depletion during both low and high-intensity exercise. These findings indicated a nitrate-mediated improvement in contractile efficiency but the mechanistic explanation was still unclear. However, NO has been shown to reduce Ca^{2+}-ATPase activity (Ishii et al., 1998) and slow myosin cycling kinetics (Evangelista et al., 2010) that might offer explanations for the lower oxygen cost. Improved mitochondrial efficiency by dietary nitrate was also a candidate to explain the lower oxygen cost, which was the subject of investigation in the first paper of this thesis.

AIMS

The general objective of this thesis was to investigate the effects of dietary nitrate on mitochondrial efficiency and metabolism in humans.

The specific aims were

- To investigate the effect of dietary nitrate on human mitochondrial efficiency (paper I)

- To investigate the effect of dietary nitrate on Ca^{2+} handling and contractile function in fast- and slow-twitch skeletal muscles in mice (paper II)

- To examine the effect of dietary nitrate on cardiovascular variables and pulmonary oxygen uptake in a human model of systemic hypoxia by prolonged breath-holding (paper III)

- To investigate the effect of dietary nitrate on resting metabolic rate in humans (paper IV).

- To explore whether metabolic efficiency has an impact on exercise performance in hypoxia (paper V).

- To investigate if cytochrome c oxidase subunit isoform COX IV-2 is present in human skeletal muscle and if so, its involvement in regulation of resting metabolic rate (paper VI).

MATERIALS AND METHODS

A brief summary of the methods used in this thesis is presented below. The reader is referred to each specific paper for a more detailed description.

Subjects

In all studies except for study II where animals were used, healthy medium- to well-trained subjects were recruited. For study III, trained breath-hold divers were used in order to be able to do measurements during prolonged apnea.

Animals

In study II, fast-twitch EDL and slow-twitch soleus muscles were isolated from 45 male C57bl/6 mice. All experiments were approved by the Stockholm North Ethical Committee on Animal Experiments.

Nitrate supplementation and dietary restrictions

Humans (paper I, III and IV)

The subjects in study I and IV were given either sodium nitrate (0.1 mmol kg^{-1} day^{-1}, divided in three doses) corresponding to a daily ingestion of 200–300 g of spinach or three to four beetroots. An equimolar amount of sodium chloride (placebo) was administered during control conditions (Potassium nitrate and potassium cloride in study III). Supplementation lasted for 3 days prior to experiments with a washout period of at least 6 days between experimentation.

Mice (paper II)

Mice were supplemented with nitrate dissolved in distilled drinking water for 7 days (1 mM NaNO$_3$ (~3.75 μmol nitrate day^{-1}) and the matched controls were provided nitrate-free distilled water. The chow contained 0.2 μmol nitrate g^{-1} and mice ingested ~0.75 μmol nitrate day^{-1} from food.

Nitrite infusion (paper IV)

Nitrite was diluted in 0.9% sterile saline solution (30 mg nitrite/ml H$_2$O) and infused in the antecubital vein at 3 different rates (1, 10, and 30 nmol · kg^{-1} · min^{-1}).

Euglycemic clamp (paper IV)

Dextrose (20%, Fresenius Kabi) was infused together with Insulin (Human Actrapid; Novo Nordisk) at a constant rate of 20 mU · m^{-2} body surface area· min^{-1} into the left antecubital vein. The infusion rate of glucose was adjusted to maintain a constant concentration of 5 mmol/L.

Oral-glucose-tolerance test (paper IV)

75g glucose was dissolved in water and consumed within 5 minutes. Following ingestion, venous blood samples were drawn every 15 min during 2 hours for blood

glucose analysis with a portable blood glucose analyser (HemoCue; HemoCue AB).

Blood and saliva sampling

Blood samples were immediately centrifuged (700 g x 10 min, 2°C) and plasma was stored at −80°C until analysed. Saliva samples were collected in Eppendorf tubes (1.5 ml) and immediately stored at −80°C.

Nitrate and nitrite in plasma

Determination of nitrate and nitrite concentration in plasma and saliva were performed with two different methods.

HPLC

Nitrite and nitrate levels were measured by high performance liquid chromatography (HPLC), (ENO-20 Eicom Japan). The system uses reverse phase chromatography to separate nitrite from nitrate. Nitrate was then reduced to nitrite in a reaction with cadmium and reduced copper inside a reduction column. Reduced nitrite was then derivatized with Griess reagent and a detector at 540 nm was used to measure the level of diazo compounds .

Chemiluminescence

In addition to the technique with HPLC, nitrate and nitrite were determined by chemiluminescence after triiodide reduction of nitrate and nitrite to NO.
Samples were introduced into a purged reaction vessel containing the reducing solution and coupled to a condenser (Sievers, Boulder, Co., USA). A constant flow of nitrogen served as the carrier gas for NO. The gas was bubbled through sodium hydroxide (1M 0°C) to trap any reminding traces of acid prior to introduction into the NO analyzer (Eco Physics CLF 77AM, Dürnten, Switzerland). The data obtained were analyzed (Windows Azur platform) and the levels of nitrite and nitrate were calculated by comparing the areas under the curve to known concentration of nitrite or nitrate.

Contractile function and $[Ca^{2+}]_i$ release in intact muscles (Study II)

EDL and soleus muscles were isolated from control and nitrate treated mice. Suture thread was used to tie stainless steel hooks to the ends of the muscles. The muscle was mounted in a stimulation chamber containing Tyrode solution (mM): NaCl, 121; KCl, 5.0; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.4, $NaHCO_3$, 24.0; EDTA, 0.1; glucose, 5.5 and fetal calf serum (0.2%). All experiments were performed at room temperature (24–26°C). Muscle was equilibrated in tyrode solution during 45 min while bubbling the solution with 95% O_2 and 5% CO_2. Supramaximal current pulses were delivered via platinum electrodes in order to stimulate tetanic contractions. One contraction/min was used to evaluate the force-frequency relationship. Durations of contractions were 300 ms and 1 s for EDL and soleus, respectively. Peak values for tetanic force were used for statistics.

$[Ca^{2+}]_i$ measurements in dissected fibres

Single flexor digitorum brevis (FDB) fibres were dissected as previously described

(Lännergren and Westerblad, 1987). The isolated fibers were mounted and tetanic stimulation was performed identical to the contractile function experiments (see above). Duration of supramaximal current pulses was set to 0.5 ms. Indo-1 (Invitrogen, Carlsbad, CA, USA) was microinjected into the muscle fibre. The fluorescence of Indo-1 was converted to free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) using an established calibration curve (Andrade et al. 1998a). The mean Indo-1 fluorescence during the last 200 ms prior to tetanic stimulation was measured as the basal $[Ca^{2+}]_i$. whereas tetanic $[Ca^{2+}]_i$ was measured as mean Indo-1 fluorescence during tetanic stimulation. Tetanic force was measured as mean maximal force over 100 ms. Force $[Ca^{2+}]_i$ relation was measured by individual 350 ms trains at frequencies ranging 15-150 Hz at 1 min intervals. From these contractions, force $[Ca^{2+}]_i$ curves were constructed. The amount of releasable Ca^{2+} stored within the sarcoplasmic reticulum was measured in another set of fibres. Tyrode solution containing 5mM caffeine was applied 1 min before stimulation (100Hz).

Exercise protocols

Study 1

Exercise tests were made on a cycle ergometer (Monark 839E, Varberg,Sweden). Maximal exercise test was performed to measure VO$_2$peak. For submaximal exercise tests, workload was chosen based on the fitness level (100, 120 and 150W). Subjects cycled until steady-state oxygen consumption ($\approx$10min). A Polar heart rate monitor was used to measure heart rate (Polar Electro, Finland).

Study III
Upon inhalation, subjects held their breath while they cycled at 50 W (60rpm) during 60s and 90s followed by maximum durations apneas. Each performance was completed twice. After the last maximum duration breath-hold, subjects continued to cycle for 10 min followed by 2 more maximum duration apneas during steady state cycling.

Study IV

Subjects were sitting on the ergometer (SRM, GmbH, Jülich, Germany) for 5 min followed by submaximal cycling at 100 and 150W alternating between a cadence of 60 and 90 rpm for 5 min each. After a short rest, a maximal exercise test was performed.

Breath holding protocols (Paper III)

Subjects performed two initial "warm-up" apneas in order to avoid warm up effects.

Static apnea protocol

Subjects' vital capacity was determined in the supine position using a hand held spirometer and the residual volume was determined by using the nitrogen dilution method. After 1 min of pre-ventilation, subjects held their breath at 0, 2 and 4 min followed by maximum effort apneas. Each breath hold was performed twice as follows: After a complete exhalation where the end tidal PCO$_2$ and PO$_2$ was measured, the subject inhaled dry air from a pre-filled anaesthetic bag corresponding to 80% of their vital capacity. At apneas longer than 3 min, the subjects were informed every 30 s until termination. Upon termination, the subjects exhaled maximally in order to measure

exhaled volume and end tidal PO_2 and PCO_2 post apnea. Blood samples were collected at baseline and 10 min after the last apnea performance. The subjects were allowed to rest for approximately 5 min between apneas. Blood pressure was measured continuously with the photoplethysmographic finger-cuff method (Finapress, Ohmeda 2300, Englewood, CO).

Dynamic apnea protocol

Vital capacity and residual volume was measured while sitting on the ergometer.
Identical preparation and breathing protocol was done in the dynamic as well as the static apneas. Immediately at the end of inhalation, the subjects initiated cycling at 50 W (60 rpm). In the first part, subjects held their breath 60 and 90 s followed by a maximum duration apnea while cycling. All breath holds were performed twice. In the second part, the participants continued to cycle for 10 min and did two more maximum duration apneas during steady state cycling. Blood pressure was measured continuously with the photoplethysmographic finger-cuff method (CNAP™ monitor 500, CNS systems. Interface, TCI105 and module DA100C, Biopac systems).

Pulmonary oxygen uptake during apnea

The volume of oxygen in the lungs was assessed prior to and at the end of each apnea. The difference in volume of oxygen corresponded to the pulmonary oxygen uptake. Oxygen content in the lungs prior to apnea was calculated from end tidal FO_2 after maximal exhalation and the known residual volume together with the FO_2 in the inhaled pre determined volume of dry air. Post apnea oxygen content in the lungs was determined through end tidal FO_2 and end apnea gas volume was calculated from the change in N_2 fraction through the assumption that the fractional volume of N_2 remained constant. All obtained volumes were adjusted to standard temperature and pressure (STPD) (25°C).

VO_2 during exercise

Oxygen uptake during exercise was measured by using a computerized metabolic system (Jaeger Oxycon Pro, Hoechberg, Germany). A flowmeter was connected to a facemask and gases were analysed breath by breath. In study V: To measure VO_2 during exercise in hypoxia, subjects breathed gas from an equilibration bag connected to a pressurized gas bottle (16% O_2). A humidifying system was connected between the gas bottle and the equilibration bag. The mouthpiece was connected to one-way valves in the direction equilibrium bag to the mouthpiece and further to the Douglas bag. The volume of the exhaled air was determined and analysed for pO_2 and pCO_2 and temperature according to the Douglas bag method.

Resting metabolic rate

An indirect calorimetric system was used to measure resting metabolic rate (Jaeger Oxycon Pro, Hoechberg, Germany). The system was connected to a ventilated hood. The measurements took place during approximately 30 min. The lowest average steady state oxygen consumption during 10 min was used as a measure of resting metabolic rate.

Muscle biopsies

Lidocain (without epinephrine) was used as anaesthetic agent of skin and muscle fascia. After a small incision at the mid section of *vastus lateralis*, muscle biopsies at a depth of 1-2 cm below fascia were obtained using a chonchotome. A small part of the biopsies were snap frozen in liquid nitrogen and stored in -80°C until analysed for protein expression or enzymatic activity. The rest were immediately placed in ice-cold mitochondrial isolation medium described below.

Mitochondrial isolation

Immediately after muscle tissue extraction, the sample was weighed and added to ice-cold isolation medium consisting of 100 mM sucrose, 100 mM KCl, 50 mM Trizma hydrochloride, 1 mM KH_2PO_4, 0.1 mM EGTA, and 0.2% BSA (weight). The sample was minced with a pair of scissors during 5 min and left for sedimentation, whereupon supernatant was removed and new isolation medium was added (1 ml). The procedure was repeated twice followed by adding isolation medium containing 0.2 mg · ml^{-1} bacterial protease (1 ml). The sample was left for 30 s on ice and vortexed at a low intensity for 30 s. The protocol was repeated once more (2 min total). The sample was further homogenized at 60 rpm in a water-cooled glass jacket. The homogenized sample was transferred to a Falcon tube containing 3 ml isolation medium and centrifuged at 700 g for 10 min (4°C). The mitochondria containing supernatant was transferred to 1.5-ml Eppendorf tubes and centrifuged at 10,000 g for 10 min. The pellet was carefully washed in isolation medium, and the buffy coat of extra mitochondrial debris was removed. Mitochondrial pellet was then resuspended and recentrifuged at 7000 g for 5 min. The pellet was then washed again and diluted in 0.6 μl preservation medium/mg tissue sample (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6\ H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, 20 mM histidine, 20 μM vitamin E succinate, 3 mM glutathione, 1 μM leupeptin, 2 mM glutamate, 2 mM malate, and 2 mM Mg-ATP). Mitochondria were left on ice for stabilization during approximately 30 minutes before the commencement of respirometric analysis.

High resolution respirometry

In study I, IV, V and VI high-resolution respirometry was performed on isolated mitochondria and cells in a 2-channel titration injection respirometer (Oroboros Oxygraph, O-2K, Austria) at 37°C using respiration medium (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6\ H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA). Speed of magnetic stirrers was set to 750 rpm. Time constants for mixing of liquid in chambers were obtained by briefly stopping and starting the stirrers and analysed in the software (Datlab 2, Oroboros, Austria). A separate background experiment was performed to estimate back diffusion of oxygen into the chamber by using at least 5 different oxygen tensions from which a linear function of oxygen tension was calculated.

Mitochondrial oxygen affinity

Mitochondrial apparent Km for oxygen (p50$_{mito}$) was determined by high resolution respirometry described above. Pyruvate (5 mM) and malate (2 mM) was used as substrates. State 3 respiration was initiated with ADP (2.5 mM) and mitochondria were

allowed to respire until anoxia. The raw signal was analysed in software Datlab2 (Oroboros, Austria) where an 8-point hyperbolic curve is fitted to the oxygen flux from which the p50$_{mito}$ was calculated.

P/O ratio

P/O ratio was determined in the presence of ATP (2 mM) by steady-state infusion of non-saturating levels of ADP with a Hamilton syringe connected to a pump (Tip-2K, Oroboros, Austria). This method has been described in detail elsewhere (Gnaiger et al., 2000). The ADP infusion rate was set to approximately 50% of maximal state 3 respiration and was performed at an oxygen tension of approximately 10 kPa. P/O ratio was calculated as the rate of infused ADP divided by the oxygen consumed during steady state upon stabilization of the respiration rate. Correction was made for the amount of oxygen added to the respiration medium by the infused ADP solution. Concentration of ADP in the infused ADP solution was determined by spectrophotometry.

Thermodynamic coupling of mitochondria

The degree of thermodynamic coupling (q) can be calculated by the equation below (Cairns et al., 1998)

$$q = \sqrt{(1 - (static\ head/state\ 3u))}$$

The static head condition corresponds to the respiration while blocking the exchange of ADP and ATP through adenylate nucleotide translocator (ANT) inhibition with 0.5 mM atractyloside. The remaining oxygen consumption is then mainly driven by proton leak. State 3u is fully uncoupled respiration during state 3 and obtained by titrating optimal concentrations of FCCP. In this state the frictional forces are minimized and the respiration can therefore proceed at an unrestricted maximum rate. The q-value reflects the degree of thermodynamic coupling where the values in different rat organs ranges from 0.88-0.97 (Cairns et al., 1998).

Mitochondrial ATP production

In study I, maximal ATP production was assessed luminometrically (pH 6.7) previously described (Wibom et al., 2002). Isolated mitochondria were diluted 1:62.5 in respiration medium (0.5 mM EGTA, 3 mM MgCl$_2$·6 H$_2$O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA). Pyruvate (5 mM) and malate (2 mM) was added as substrates. p1,p5-diadenosine-5-pentaphospate (DAPP) was used as ATP:AMP phosphotransferase inhibitor. The reaction was initiated by the solution containing ADP, AMP, and DAPP. The analog signals from the luminometer (1251 Bio Orbit Oy, Turku, Finland) were sampled at 200 Hz by using an A/D converter (MP150 BioPac Systems, Inc.) and subsequently analyzed in Acqknowledge 3.7.3 software.

Western blotting

Protein concentration was determined in muscle/cell homogenates with the BCA method and adjusted to the same concentration before dissolved in Laemmli sample buffer containing 4% 2-mercaptoethanol and denatured by heating to 95°C for 5 min. Protein separation was performed by SDS-PAGE in polyacrylamide gradient gels (4-

20%) at 175V. Proteins were transferred to PVDF membranes for 90 min at a constant current of 300 mA (4°C) followed by blocking with TBS-T 5% milk for 1 hour followed by incubation with primary antibodies over night at 4°C. Primary and secondary antibodies were diluted in TBS-T 2.5% milk.

Antibodies used in study I: UCP3; Chemicon 1:500, AB3046. ANT-1; 1:200, No.sc-9300, Santa Cruz. Secondary antibody: No.sc-2030, Santa Cruz.

Antibodies used in study II: SERCA1; 1:2500, no. ab2818, Abcam. CASQ; 1:2500, no. ab3516, Abcam, DHPR; 1:500, no. ab2864, Abcam, RyR, 1:1000, MA3-925, Thermo Scientific. Secondary antibodies: IRDye 680-conjugated goat anti-mouse IgG and IRDye 800-conjugated goat anti-rabbit IgG (1:15,000, LI-COR) in blocking buffer and 0.01% SDS.

Antibodies used in study VI: COX IV-2; 1:1000, H00084701-M01, Abnova, COX IV-2; HPA029307, Atlas antibodies, COX IV-1; 1:1000, #4850, Cell signalling, Citrate Synthetase; 1:1000, ab96600, abcam. Secondary antibodies: anti-mouse; 1:10000, HRP-linked antibody 7076, Cell signalling, anti-rabbit; 1:10000 HRP-linked antibody 7074, Cell signalling.

Visualisation of bands

In study I, immunoreactive bands were visualized by chemiluminescent detection with a Molecular Imager ChemiDoc XRS system. All bands were quantified using the contour tool in the Quantity One version 4.6.3 software (Bio-Rad Laboratories)
In study II, Immunoreactive bands were visualized using infrared fluorescence (IR-Odyssey scanner, LI-COR)
In Study VI, Chemidoc ™ MP imaging system, Bio-Rad was used for visualization and bands were quantified using software Image lab 5.0 (Bio-Rad laboratories)

Real time PCR

In study I, gene expression of COX IV, PGC-1α and TFAM were measured in human muscle biopsies. RNA was extracted from freeze-dried tissues with a power homogenizer (KEBO-lab) and TRIzol reagent. RNA was reverse transcribed (1 μg) using M-MLV reverse transcriptase. Real time PCR was used to determine the relative expression levels of each gene (7900 sequence-detection system, Applied Biosystems). Nuclear and mitochondrial DNA was extracted with DNAesy blood & tissue Kit (Qiagen) following the manufacturers instructions. Mitochondrial and nuclear DNA quantification was performed by multiplex real-time PCR using mtDNA qkit (Genemore Italy srl, Modena, Italy). Signal obtained from probes for mtDNA and nDNA was detected with FAM and Texas filters respectively. ABI PRISM 7500 sequence Detector (Applied Biosystems) was used for detection and amplification.

Body composition

Body fat was determined by the 7-site skin fold method. Obtained values were put in the online skin fold calculator: http://www.exrx.net/Calculators/BodyComp.html.

Enzymatic activity

Citrate synthase activity was obtained from isolated mitochondrial samples and homogenates from freeze-dried biopsies at 25°C described previously (Reisch and Elpeleg, 2007). A commercial kit was used to determine Cytochrome c oxidase activity (CYTOCOX1, Sigma) following the manufacturers instructions.

Cell transfection

Co-transfection of differentiated cells was performed in order to achieve overexpression and knock down of COX IV-2 and COX IV-1 respectively. Cells were grown in T-75 flasks until approximately 80% confluence. Following differentiation, cells were co-transfected with an expression vector and siRNA simultaneously (Expression vector: human cDNA Clone, NM_032609, vector CMV6XL5, Origene, siRNA : ON-TARGETplus siRNA, L-011625-01, Dharmacon). CO-transfections were performed according to the manufacturers instructions (DharmaFECT Duo Transfection Reagent, Thermo SCIENTIFIC). In order to efficiently knock down COX IV-1, the protocol had to be repeated at day 3 after transfection. Cells were harvested at the 6th day. Successful transfection and knock down was verified by Western blotting. Control cells were treated accordingly: Plasmid, CMV6XL5 empty vector, Origene, siRNA: ON-TARGETplus Non-targeting Control siRNA # D-001810-01, Dharmacon.

Plasmid cloning

Plasmids were amplified in competent cells (Library Efficiency® DH5α™ Competent Cells, Invitrogen, life technologies). 5 ng plasmid dissolved in distilled water was added to 50 ul freshly thawed bugs and incubated on ice for 25min followed by heat shock during 60s (42°C). While placed on ice, 600 µl ice-cold SOC medium (Invitrogen) was added to the cells immediately after the heat shock. The solution was incubated under agitation at 37°C for 60 min. A small part of the solution was plated out on agar plates containing Amphicillin (100 µg/ml) for selection of transformed cells. 2-3 bacterial colonies were inoculated into sterile culture tubes with LB-medium containing Amphicillin (100 µg/ml). Isolation of DNA was done with a commercial kit (Plasmid midi kit, QIAGEN) following the instructions of the manufacturer. The amplified plasmid was stored in TE-buffer (10 mM TRIZMA base, 1 mM EDTA, adjusted to pH 8.0 with HCl) at -20°C. The yield was determined spectrophotometrically (Nanodrop 1000, Thermo Scientific).

Isolation of human myogenic satellite cells (paper V and VI)

Biopsies were stored over night in PBS (4°C). Sample was cleaned from visible connective tissue and washed in PBS. After addition of a few drops of trypsin (0.25%, 1 mM EDTA), tissue was minced with a pair of scissors. The sample was placed in a small beaker with ≈ 5 ml trypsin and put in an incubator under gentle agitation for 20 min (37°C, 5% CO_2). After 5 min sedimentation, supernatant was collected in growth medium (50% Dulbecco's modified Eagle's medium (DMEM-F-12) and 50 U/ml penicillin, 50 µg/ml streptomycin, 1,25 µg/ml Amphotericin containing 20% fetal bovine serum (FBS)) followed by centrifugation for 5 min at 1500 rpm 5 min (Sigma, 1A). Cells were resuspended in growth medium and plated on a petri dish for 30 min.

Cells still in suspension were then re-plated in T25 flasks (Sarstedt, Stockholm, Sweden) and transferred to T75 flasks upon confluence.

Cell culture

Cells were cultured in T75 S Cell$^+$ growth surface flasks (Sarstedt) in a humidified atmosphere at 37°C (5% CO_2). F12 (Gibco) and Dulbecco's modified Eagle's medium (DMEM, Gibco) at a ratio of 1:1, 50 U/ml streptomycin, 50 U/ml Penicillin, and 1.25 ug/ml Amphotericin. (20% FBS) was used as a cell culture medium. For differentiation of the cells, FBS content was reduced to 2%.

Cell preparation for respirometric analysis

Cells were treated with trypsin and resuspended in cell medium and centrifuged at 1500 rpm for 5 min (Sigma A1). Supernatant was discarded and the pellet washed in PBS and re-centrifuged. Cellular pellet was then resuspended in cell respiration medium (EGTA 0.5 mM, MgCl2 3 mM, K-lactobionate 60 mM, Taurine 20 mM, KH_2PO_4 10 mM HEPES 20 mM, Sucrose 110 mM, BSA 1g/l, DTT 0.3 mM). Trypan blue 0.4% in respiration medium 1:1 was used to determine cell viability. Cells were counted manually with a hemocytometer.

Cell respiration

High resolution respirometry (O2-K, Oroboros) was used for measuring cell respiration. Initially, basal cell respiration was measured followed by permeabilization with digitonin. Saturated levels of pyruvate, malate and succinate were used as substrates. Respiration was measured both in absence and presence of ATP (5 mM).

Harvesting of cells for immunoblot analysis

Cells were washed with ice cold PBS twice and collected by adding 1.5 ml PBS to the flask and further detached with a cell scraper. Following centrifugation at 3000 rpm 5 min 4°C (Sorvall, Legend micro 17r, Thermo Scientific), pellet was resuspended in RIPA buffer (50 mM Tris-HCl pH 7,4, 1% NP-40, 0,5% deoxycholate, 0,1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 μl/ml protease inhibitor cocktail (Sigma-Aldrich, P-8340). Samples were placed on ice and wortexed every 10 min during 30 min and then sonicated during 3 sec (MSE, Soniprep 150). After centrifugation at 14000 rpm (Sorvall, Legend micro 17r, Thermo Scientific), supernatant was collected and kept at -80°C until analysed.

Protein concentration determination

The BCA method was used for all protein concentration determinations (Micro BCA protein assay kit, Thermo scientific). Samples were analysed in spectrophotometer (SPECTRAmax PLUS384 Microplate reader, Molecular Devices)

H_2O_2 production in cells

O2k-Fluorescence LED2-Module was connected to the oxygraph (Oroboros, oxygraph Innsbruck, Austria) and Amplex® UltraRed Reagent at a final concentration of 10 μM (10 mM stock solution diluted in DMSO, Sigma D8418) together with horseradish peroxidase (1 U/ml) (Sigma-Aldrich P 8250). H_2O_2 production was measured

simultaneously with oxygen consumption (37°C). Calibration was done by stepwise titrating 5 μl of a H_2O_2 stock solution (40 μM, Sigma-Aldrich 323381, 10 μM HCl) corresponding to a step increase of 0.1 μM H_2O_2. The H_2O_2 -signal was subsequently analysed in Datlab 5.

Hydrogen peroxide challenge

Cells were cultured and differentiated in 12-well plates before co-transfected as previously described. Cells were treated with a bolus dose of different concentrations of H_2O_2 (Sigma-Aldrich 323381). Viability (Trypan blue 0.4% in respiration medium 1:1) and cell number in each well was determined and counted manually (hemocytometer).

Cells exposed to hypoxia and chemical induced hypoxia

Cells cultured in T75 flasks were differentiated and placed in a hypoxia chamber (Whitley H35 Hypoxistation, Don Whitley Scientific) during 24 h (1.0 and 0.1) % O_2. In another set of cells, $CoCl_2$ **(250 μM)** was used for chemically inducing hypoxia during 24 h.

Statistics
Results are expressed as mean ±SEM. P-values <0.05 were considered significant. Student's paired t-test (Grafpad Prism 5) and Two-way repeated measures ANOVA (SigmaStat 3.1, Systat Software, Chicago, IL, USA) were used when comparing repeated measurements. Student's unpaired t-test was used when comparing treatments between groups. Normality distribution was evaluated using D'agnostino and Pearson omnibus normality test. Wilcoxon signed rank test was used when samples were not normally distributed. Bonferroni correction was performed for multiple comparisons. Pearson r was used for correlation analysis. The software: Grafpad Prism 5 was used for all statistical analyses.

RESULTS AND DISCUSSION

Paper I

Dietary inorganic nitrate improves mitochondrial efficiency in humans

In 2007, our group discovered that dietary nitrate reduces oxygen consumption during submaximal exercise (Larsen et al., 2007). This was later confirmed in a series of subsequent studies with different set ups (reviewed in Jones et al., 2012). Therefore we wanted to investigate whether dietary nitrate contributes to changes in mitochondrial efficiency that could explain this effect. Study I is a randomized placebo-controlled, double-blind, cross-over study where the subjects were supplemented for 3 days with sodium nitrate or placebo (0.1mmol kg^{-1} bw^{-1} day^{-1}). Maximal and submaximal exercise tests were performed while measuring oxygen uptake, volume of produced carbon dioxide (VCO$_2$) and respiratory exchange ratio (RER). Biopsies were extracted from *vastus lateralis* from which we isolated mitochondria and performed high resolution respirometry.

The effect of dietary nitrate on basal mitochondrial function

A variety of basal respiratory variables were measured in isolated mitochondria harvested after nitrate administration and compared to placebo. We found that variables connected to proton leak, that ultimately affects mitochondrial efficiency, were lower after nitrate supplementation including leak- and state 4 respiration (with and without adenine nucleotide translocase inhibition with atractyloside) (Fig. 3). Maximal ADP stimulated respiration (state 3) and uncoupled respiration did not change which led to the improved calculated thermodynamic coupling.
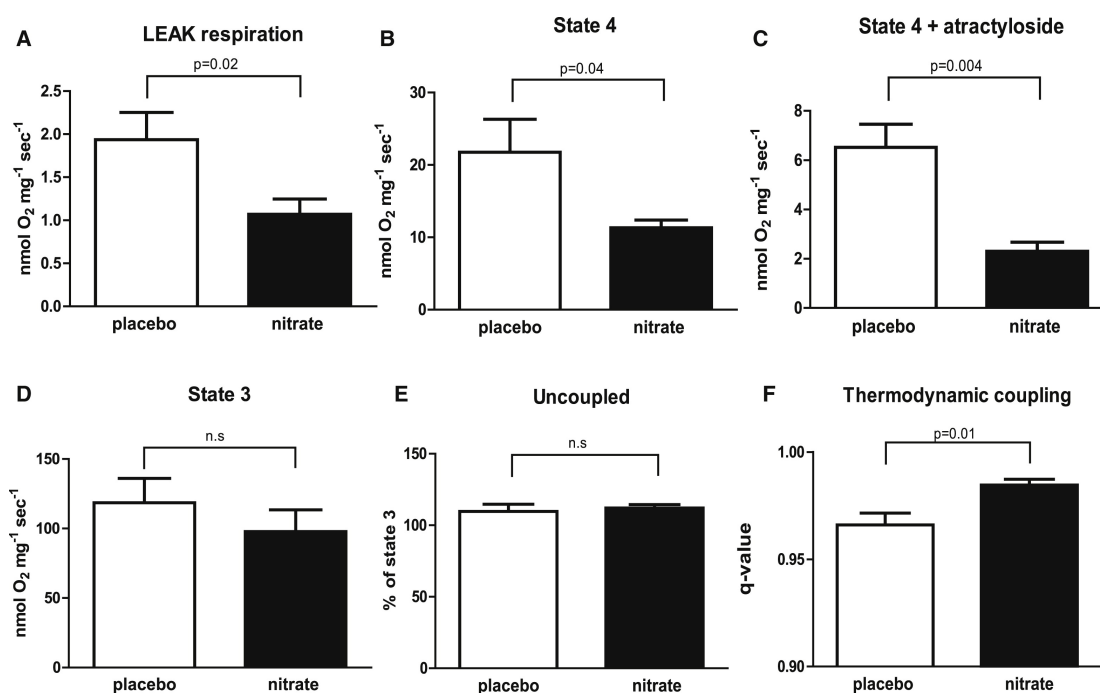
Figure 3. Mitochondrial function after 3 days of dietary nitrate supplementation or placebo. (A) LEAK respiration (n = 9), (B, C) State 4 with (n = 6) and without atractylocide (n = 14). (D) State 3 respiration and (E) FCCP uncoupled respiration (n = 6). (F) Thermodynamic coupling (q value) after dietary nitrate supplementation (n = 6). Data are mean ± SEM.

The effect of dietary nitrate on mitochondrial P/O ratio and relation to oxygen consumption

The standard method to measure mitochondrial P/O ratio is by addition of a saturated amount of ADP at ambient oxygen tensions ($\approx$ 20 kPa O_2) (Gnaiger et al., 2000). However, this method poorly reflects the *in vivo* situation. Here we wanted to investigate the effect of dietary nitrate on mitochondrial P/O ratio in a way that better resembles the conditions during submaximal exercise (Willis and Jackman, 1994). Therefore we set the respiration medium to pH 6.7 (Sahlin et al., 1978) and performed a steady state infusion of non-saturating levels of ADP. Three days of sodium nitrate supplementation improved P/O ratio by 19% (Fig. 4). In addition, the improvement in P/O ratio correlated inversely with the reduction in whole body oxygen consumption during submaximal exercise. These data suggest that improved mitochondrial efficiency by dietary nitrate undelies the reduction in oxygen consumption during exercise.
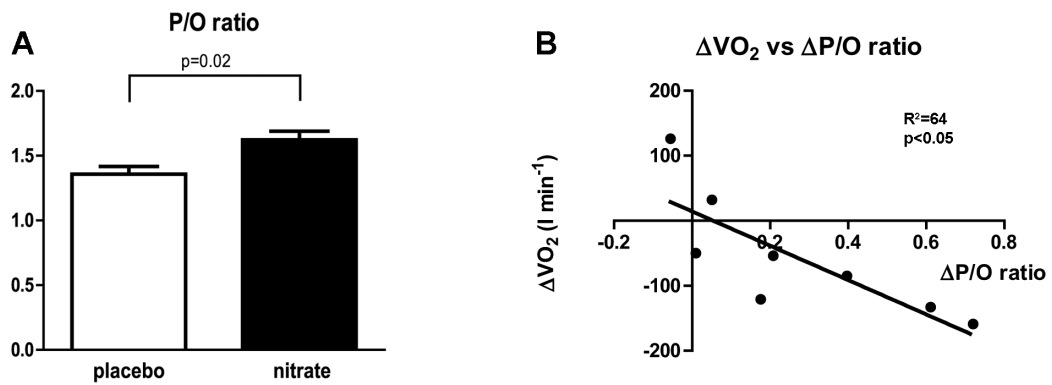
Figure 4. (A) Mitochondrial P/O ratio after 3 days of nitrate administration or placebo (0.1mmol kg^{-1} bw^{-1} day^{-1}). (B) Relationship between change in mitochondrial P/O ratio and change in whole body oxygen consumption during submaximal exercise after nitrate intake.

The effect of dietary nitrate on uncoupling proteins

The respirometric results suggested that mitochondrial efficiency was improved as a result of a decreased proton conductance. Therefore, we went on to investigate two proteins previously implicated in uncoupling of oxidative phosphorylation; adenine nucleotide translocase (ANT) and uncoupling protein 3 (UCP-3) (Bevilacqua et al., 2010; Parker et al., 2008). Western blot analysis of *vastus lateralis* skeletal muscle showed that levels of ANT were significantly reduced compared to placebo after nitrate intake. A similar pattern was seen for UPC-3 but due to an extreme outlier, this failed to reach statistical significance (Fig. 5).
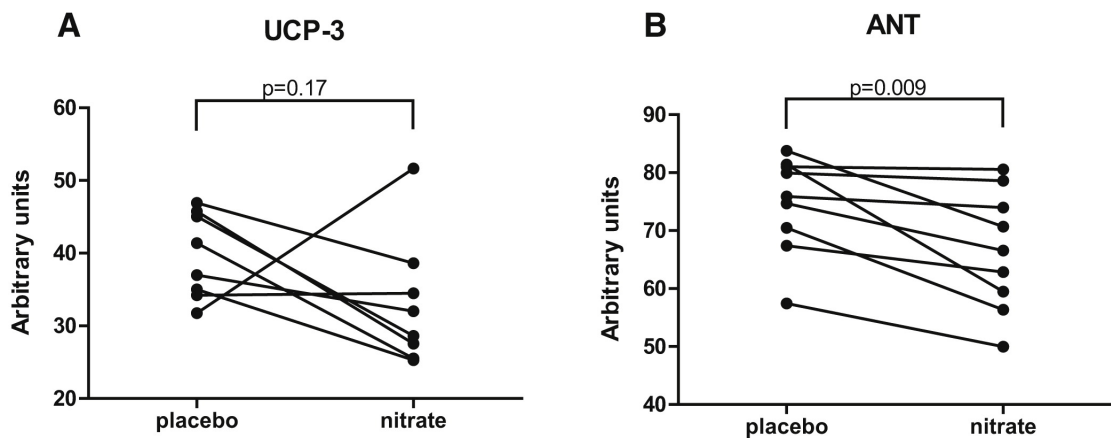
Figure 5. (A) Western blots performed on skeletal muscle (*vastus lateralis*) after 3 days of nitrate supplementation (0.1mmol kg^{-1} bw^{-1} day^{-1}) with antibodies raised against (A) UCP-3 and (B) ANT.

Oxidative phosphorylation is not fully coupled to ATP production. Increased efficiency of oxidative phosphorylation would therefore be reflected in lower whole body oxygen consumption as observed after dietary nitrate administration. Although experiments performed on isolated mitochondria are frequently criticized to poorly reflect the *in vivo* physiological state, here we were able to detect a significant

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relationship between the change in P/O ratio in isolated mitochondria and the change in the whole body variable (VO$_2$), clearly suggesting that a large part of the nitrate-induced improvement in whole body efficiency takes place at the mitochondrial level. Moreover, the correlation between P/O ratio was even stronger with the whole body efficiency variable Watt/O$_2$ consumed, further supporting this suggestion (Paper I, Supplement Fig S3). In addition to the mitochondrial effects in this study, Bailey and colleagues had shown that nitrate-mediated reduction in VO$_2$ during exercise was associated with sparing of intramuscular phosphocreatine and blunting of increases in ADP and inorganic phosphate (P$_i$), indicative of a reduced ATP cost for muscle force production after nitrate loading (Bailey et al., 2010).

Paper II

Dietary nitrate increases tetanic [Ca2$^+$]$_i$ and contractile force in mouse fast-twitch muscle

With respect to the nitrate-mediated reduction in oxygen cost during exercise, this intervention appears to have both mitochondrial (paper I) and extra-mitochondrial effects (Bailey et al., 2010). Reduced ATP cost for muscle force production had been suggested as one of the mechanisms that could contribute to lower VO$_2$ (Bailey et al., 2010; Ferreira and Behnke, 2011). In study II, contractile function and Ca^{2+} handling in skeletal muscles from mice were explored after 7 days of dietary nitrate supplementation. It was hypothesised that dietary nitrate would improve contractile performance due to increased Ca^{2+} activation of contractile proteins.

Effect of dietary nitrate on contractile force

Slow (soleus) and fast twitch muscle (EDL) were isolated from control and nitrate fed mice and subjected to electric stimulation. After tetanic stimulation at frequencies ranging between 1-150 Hz for EDL and 1-100 Hz for soleus, peak force production was measured. Tetanic force was significantly increased at stimulation frequencies below 70Hz only in EDL muscles (Fig. 6A). These findings were paralleled by higher myoplasmic free [Ca^{2+}] in nitrate-treated mice compared to controls.
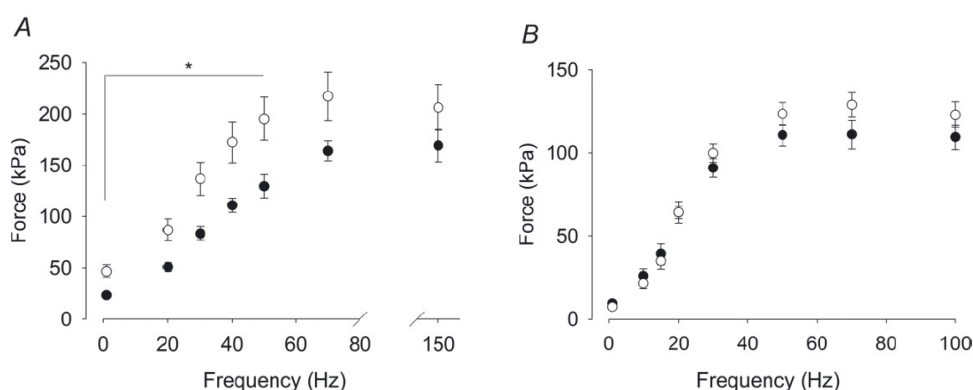
Figure 6. (A) Contractile force during tetanic stimulation in fast-twitch EDL muscles from nitrate fed mice (n=7) compared to controls, (n=7) (B) Contractile force in slow-twitch soleus muscles during tetanic stimulation (n=6). Filled symbols represent control mice and open symbols represents nitrate fed mice. Data is presented as ±SEM. *P < 0.05

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Dietary nitrate affects the expression of Ca^{2+} handling proteins

The expression of proteins involved in the regulation of Ca^{2+} release from the sarcoplasmic reticulum (SR) were investigated and the expression of CASQ1 and DHPR was significantly higher in EDL muscle of nitrate treated mice compared to controls (Fig. 7A.). The levels of RyR and sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA 1) were unchanged (Fig. 7A, C). The absence of effects on contractile force after nitrate treatment in soleus (slow-twitch) indicated that there would be no change in Ca^{2+} handling proteins in this muscle type and indeed no differences were seen (Fig. 7B). No effect of nitrate was observed on SR Ca^{2+} pumping or passive leak measured as $[Ca^{2+}]_i$ tails after stimulation at 30 and 100 Hz (Fig. 7D).
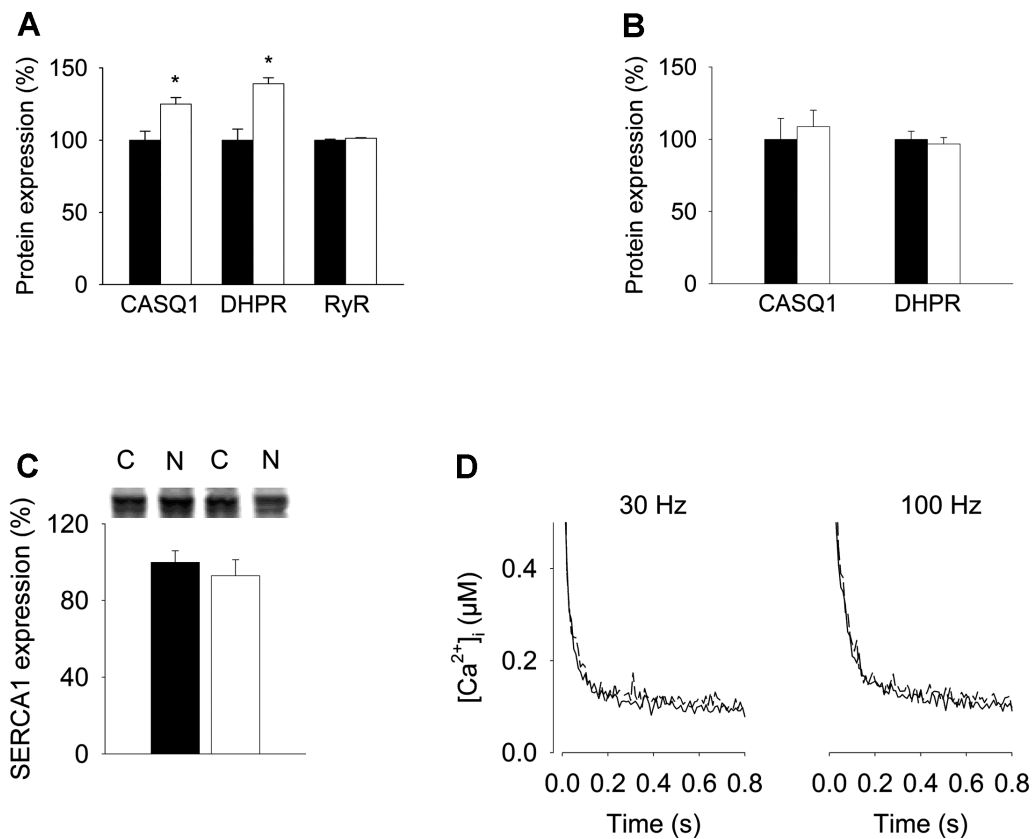
Figure 7. Western blots performed on EDL (A) and soleus (B) with antibodies raised against CASQ1, DHPR, and RyR. (C) SERCA1 expression. Control is filled bars with mean set to 100% and nitrate is open bar. *P < 0.05. (D) Average records of $[Ca^{2+}]_i$ tails after 30 and 100 Hz stimulation. Nitrate mice (n = 5, dashed line) and control (n = 7, continuous line).

The improved contractile force in fast twitch muscles together with increased tetanic $[Ca^{2+}]_i$, increased expression of Ca^{2+} handling proteins (CASQ1, DHPR) and the increased SR Ca^{2+} content suggest a targeted effect of nitrate supplementation on contractile function in fast-twitch skeletal muscle. Interestingly, the most pronounced effects were found within the 15–50 Hz frequency range, which corresponds to previously recorded frequencies from motor neurones during normal movements *in vivo* in the rat (Hennig and Lømo, 1985). Results in line with these were recently reported in humans by Haider & Folland who could show that beetroot juice (high in nitrate) improved knee extensor peak force response to low frequency electrical

stimulation (Haider and Folland, 2014). The increased expression of CASQ1 and DHPR can explain the improved contractile function. CASQ1 buffers Ca^{2+} in the SR lumen and maintain free Ca^{2+} at ~1mM (Beard et al., 2004). By increased expression of CASQ1 storage of SR Ca^{2+} is most likely increased and subsequently the release of Ca^{2+}. DHPRs are voltage sensors in skeletal muscle and form close connections with RyRs (Tanabe et al., 1990). Upon depolarization, DHPRs relay action potential activation to the RyRs that facilitates SR Ca^{2+} release (Lamb, 2000). Increased DHPR content may therefore potentially lead to greater Ca^{2+} release. The lack of effect in slow-twitch muscle is interesting and may depend on the higher content of glutathione and antioxidant enzymes compared to fast-twitch muscle (Ji et al., 1992). These properties may counteract the effects of nitrite. Moreover, the higher glycolytic activity in fast-twitch muscles compared to slow-twitch, with resulting lower pO_2 and pH, will favour nitrite reduction to NO.

Impaired Ca^{2+} handling due to reduced CASQ1 expression has been connected to diseases with muscle weakness (Aydin et al., 2009). Recently, Coggan et al. observed a positive effect on voluntary maximal knee extensor power and velocity in humans even after an acute dose of beetroot juice (Coggan et al., 2014). This effect is obviously not related to differences in protein expression but may instead be related to cGMP mediated phosphorylation of myosin regulatory light chain (Maréchal and Gailly, 1999). Thus, dietary nitrate has a variety of positive effects on muscular function and may therefore have a potential for future treatment of skeletal muscle pathology.

Paper III

Effects of dietary inorganic nitrate on static and dynamic breath-holding in humans

The enzymatic and non-enzymatic pathways described for reduction of nitrite to NO are all enhanced during hypoxia or low pH (Lundberg et al., 2009; Weitzberg et al., 2010). This fact together with the findings that dietary nitrate improves mitochondrial function and reduces oxygen cost during exercise led us to test the effect of nitrate intake in a human model of general hypoxia. For this reason we recruited competitive and recreational breath-hold divers in a placebo-controlled cross-over study. These subjects were capable of performing apneic durations beyond 4 min. We hypothesised that nitrate intake, by reducing whole body oxygen consumption, would attenuate hypoxia during prolonged breath-holding. We measured pulmonary oxygen uptake along with arterial oxygen desaturation and cardiovascular responses during breath-holding at rest (static apnea) and during cycling ergometry (dynamic apnea). Tests were performed after 3 days of either potassium nitrate ($0.1 \ mmol \ kg^{-1} \ bw^{-1} \ day^{-1}$) or potassium chloride (placebo).

The effect of dietary nitrate on oxygen consumption during static apnea

Contrary to our hypothesis, dietary nitrate increased the rate of arterial oxygen desaturation during static apnea, leading to lower oxygen saturation at 4 min of apnea as well as at the end of the maximum effort apneas compared to placebo (Fig. 8A). The lower arterial oxygen saturation was accompanied by lower exhaled fraction of oxygen after 4 min (Fig. 8B). In addition, the maximum duration apneas were significantly shorter after nitrate administration (Fig. 8C).

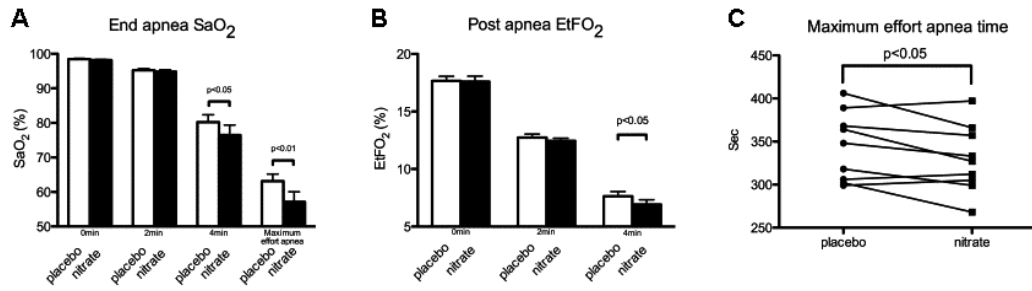During ergometer cycle exercise dietary nitrate did not affect any of the variables related to oxygen consumption.

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Figure 8. (A) Arterial oxygen saturation during static apnea at different time points. (B) Post static apnea end tidal fraction of oxygen. (C) Maximum apnea durations after nitrate and placebo.

The effect of dietary nitrate on the diving response

The diving response is an oxygen conserving mechanism and consists of vagal-induced bradycardia and sympathetic-induced peripheral vasoconstriction that generates hypertension during apnea (Lindholm and Lundgren, 2009). During the maximum duration dynamic apneas dietary nitrate attenuated the reduction in heart rate and showed a tendency to attenuate also the increase in mean arterial blood pressure (Fig. 9 A, B).
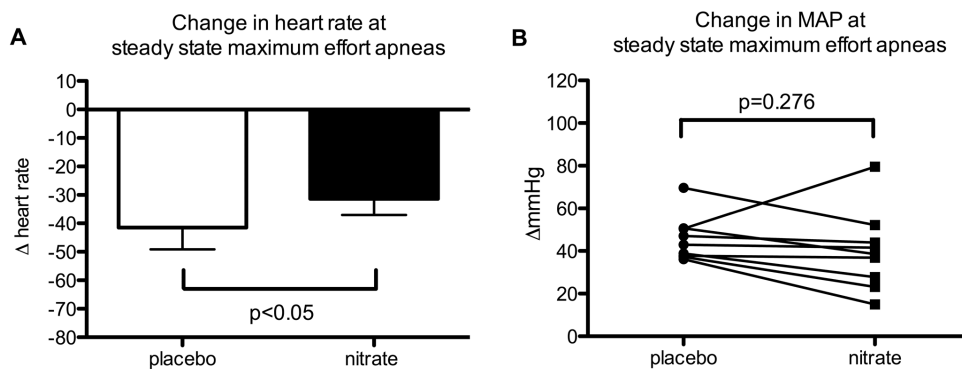
Figure 9. (A) Change in heart rate and (B) mean arterial blood pressure during maximum effort steady state cycling apneas.

Contrary to our hypothesis that nitrate would reduce oxygen consumption and thereby improve oxygenation during general hypoxia, dietary nitrate actually increased pulmonary oxygen uptake during static apnea. Nitrate lowered arterial oxygen saturation, reduced end-apnea exhaled fraction of oxygen and led to shorter maximal apneas. In contrast, Engan et al. have reported prolonged breath-hold times after acute administration of beetroot juice (Engan et al., 2012). However, several methodological differences could explain these diverging results and are thoroughly discussed in paper III. The indications of enhanced oxygen uptake during static apnea after nitrate supplementation may possibly be explained by a NO-mediated attenuation of the diving response. This was indicated by the reduced bradycardia and attenuated increase in blood pressure, measured during dynamic apneas. The diving response has previously been shown to be proportional to oxygen sparing by delaying the arterial and pulmonary gas exchange during apnea (Andersson et al., 2008; Lindholm and

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Linnarsson, 2002; Lindholm et al., 1999). Fuelling the nitrate-nitrite-NO pathway may have led to NO-mediated microcirculatory vasodilation, resulting in greater arterial oxygen desaturation. Although nitrate administration had negative effects on oxygen saturation and breath-hold performance in this human model of general hypoxia it has been shown that dietary nitrate improves exercise tolerance in conditions with decreased peripheral blood flow and hypoxia such as peripheral arterial disease (Kenjale et al., 2011).

Paper IV

Dietary nitrate reduces resting metabolic rate in humans

The resting metabolic rate (RMR) is the minimum energy required to sustain vital body functions in a resting state during fasting conditions. In paper I, we showed that dietary nitrate reduces oxygen cost during exercise by improving mitochondrial efficiency and downregulation of proteins involved in proton conductance. We wanted to investigate if similar effects would be achieved also on RMR. In a randomized, placebo-controlled, crossover study healthy volunteers were supplemented with nitrate or placebo (0.1 mmol kg^{-1} bw^{-1} day^{-1}) for 3 days and the effects on RMR and associated variables of normal metabolic function were measured. In separate groups of healthy subjects we measured RMR during acute i.v. infusion of sodium nitrite and performed oral glucose tolerance tests and euglycemic hyperinsulinic clamps after 3 days of nitrate/placebo administration.

The effect of dietary nitrate on resting metabolic rate

Nitrate intake reduced oxygen consumption and RMR by approximately 5% and 4%, respectively compared to placebo (Fig. 10A, B). Respiratory exchange ratio was unaltered indicating no change in the utilization of substrates (glucose, fat). In contrast, acute infusion of nitrite did not change RMR in a separate group of healthy volunteers.
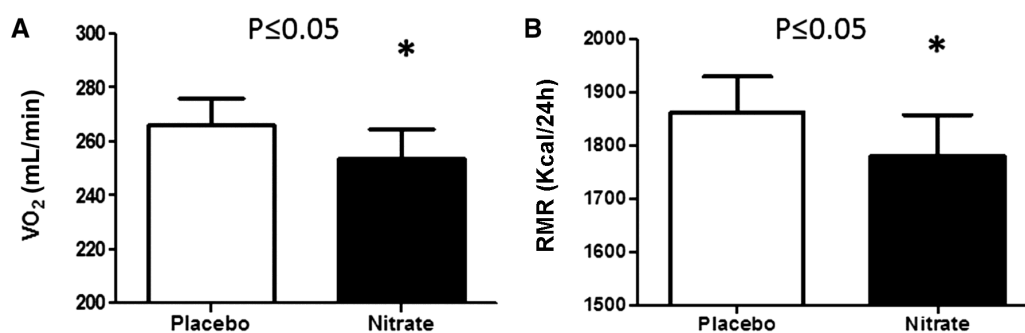
Figure 10. Measurements of (A) resting VO$_2$ and (B) resting metabolic rate after 3 days of nitrate or placebo administration (0.1 mmol kg^{-1} d^{-1}). *P < 0.05.

The effect of dietary nitrate on thyroid hormones and glucose metabolism

Thyroid hormones can affect metabolic rate and uptake of iodine in the thyroid gland is in competition with inorganic nitrate (Bloomfield et al., 1961). To exclude effects on thyroid hormone status by dietary nitrate we measured plasma levels of T3, T4 and TSH but found no effects of treatment compared to placebo. In addition, dietary nitrate had no apparent effect on glucose uptake or insulin sensitivity.

Salivary nitrate and resting metabolic rate

Nitrate is actively taken up by the salivary glands, excreted in saliva and reduced to nitrite by oral bacteria. Swallowed nitrite reaches the systemic circulation and is further reduced to NO in blood and tissues (Weitzberg and Lundberg, 2013). We found no correlations between the changes in plasma and salivary nitrite and RMR but changes in salivary nitrate showed a strong inverse correlation with changes in RMR (Fig. 11). Thus, salivary nitrate accumulation appears to be crucial for the nitrate related effect on resting metabolic rate.
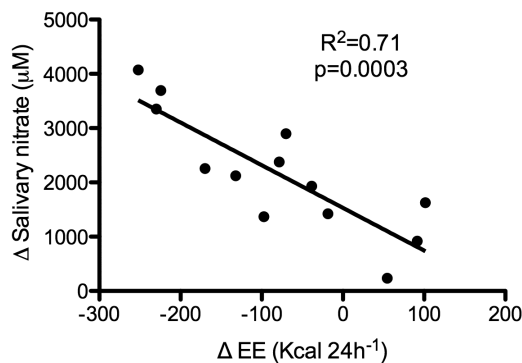
Figure 11. Correlation between nitrate-induced changes in salivary nitrate and change in energy expenditure

The effect of acute nitrite exposure in isolated primary myotubes

Satellite cells were isolated and cultured from the subjects skeletal muscle (*vastus lateralis*) and then exposed to nitrite (25 µM). Nitrite induced a 40% reduction in basal oxygen consumption compared to control cells (Fig. 12). In contrast, leak and ADP stimulated respiration were unaltered by nitrite in permeabilized cells, indicating that nitrite may have to interact with a cytosolic component that converts this anion to NO or another bioactive nitrogen species.
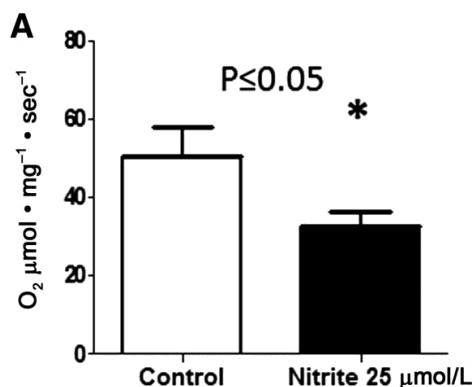
Figure 12. Basal oxygen consumption in primary myotubes before and after nitrite administration (25uM). (*P < 0.05, N=6).

The reduction in RMR by dietary nitrate was of similar magnitude as seen in VO_2 during exercise. We did not measure mitochondrial proteins involved in proton conductance in this study but with respect to the findings in paper I, it is likely that reduced proton leak may underlie the observed reduction in RMR. Since dietary nitrate did not alter thyroid hormone levels it is unlikely that a significant interference with iodine uptake and altered thyroid hormone status underlies the reduction in RMR (Pesce and Kopp, 2014; Toubro et al., 1996). The strong correlation between

nitrate-induced increase in salivary nitrate and change in RMR supports the importance of the enterosalivary circulation of nitrate previously described (Fig. 2). The reduction of basal respiration in primary myotubes after acute exposure to nitrite is obviously not related to changes in protein expression (ANT and UCP-3). It may rather be explained by NO-dependent COX inhibition which leads to improved COX efficiency (Clerc et al., 2007). By contrast, no effect on basal metabolism could be observed as a result of acute nitrite infusion although plasma nitrite levels became much higher than those achieved by dietary nitrate (see paper IV for data). However, the plasma concentrations of nitrite measured after acute nitrite infusion were lower lower than those used in the *in vitro* experiments. These data suggest that dietary nitrate-mediated changes in RMR are likely to be explained by changes in protein expression of proteins involved in mitochondrial uncoupling. A sustained reduction in RMR without a concomitant reduction in dietary intake can have implications on body-weight regulation and related cardiovascular problems. However, due to the well-established protective effect on cardiovascular disease by high intake of vegetables, it would not be appropriate to advocate a lower intake of nitrate-containing green leafy vegetables in an attempt to control weight. Interestingly, caloric restriction (CR) has a similar effect on RMR and metabolic adaptations linked to reduced risk of cardiovascular disease (Doucet et al., 2001; Heilbronn et al., 2006; Rosenbaum et al., 2008; Tremblay and Chaput, 2009). In addition, CR consistently increases life span in a wide range of animal models (Fontana et al., 2010) and future experimental studies may reveal whether also dietary nitrate may have similar effects.

Paper V

Dynamic regulation of metabolic efficiency explains tolerance to acute hypoxia in humans

In the mid 50s, Odum and Pinkerton proposed that biological systems tend to evolve and self-organize toward a degree of efficiency that maximizes power production at the cost of efficiency (Odum and Pinkerton, 1955). This is termed the "maximal power principle" and is sometimes considered as the fourth principle of energetics in thermodynamic theories. In humans, the metabolic efficiency (ME) varies between 16-23% during cycling. Rather surprising, training does not improve cycling efficiency (Mogensen et al., 2006). Several studies have demonstrated that altitude acclimatization improves exercise economy (Gore et al., 2001; Green et al., 2000; Hochachka et al., 1991; Humberstone-Gough et al., 2013; Katayama et al., 2003; Katayama et al., 2004; Marconi et al., 2005; Saunders et al., 2004; Saunders et al., 2009; Schmitt et al., 2006) while others have failed to detects improved ME following altitude exposure (Bonetti et al., 2006; Lundby et al., 2007; Truijens et al., 2008). We aimed to study whether the maximum power principle applies acutely in whole body physiology and isolated mitochondria. Our main interest was to determine whether inter-individual variation in metabolic efficiency is an important factor in tolerance to hypoxia during exercise and if mitochondrial variations between subjects could provide an explanation. Further, to examine the effects of acute hypoxia on whole body metabolic efficiency.

Whole body efficiency in relation to hypoxic tolerance during exercise

Fourteen subjects were divided into two groups according to their change in exercise capacity during hypoxia (16% O_2); large loss group (LL) and small loss group (SL). The group that had a small loss in exercise capacity during hypoxia had a

significantly higher metabolic efficiency in normoxia (Fig 13 A, B). The association between the loss of exercise capacity in hypoxia and the metabolic efficiency in normoxia is illustrated in figure 13 C.
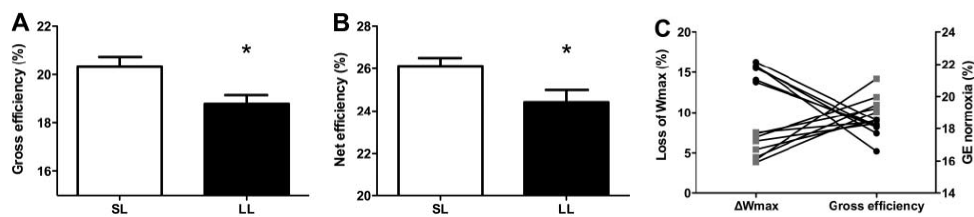

Figure 13. Comparison of (A) gross and (B) net efficiency during normoxia in subjects that better maintain exercise performance in hypoxia (small loss, SL) and those suffering from hypoxia (large loss, LL). (C) Relationship between gross efficiency in normoxia and the relative reduction of W_{max} in hypoxia. *P < 0.05.

Acute changes in metabolic efficiency during exercise in hypoxia

VO_2 is usually used in studies as a crude estimate of metabolic efficiency. Regarding the effects of moderate hypoxia on exercise VO_2 conflicting results exist, with both unchanged (Fukuda et al., 2010; Lador et al., 2013) or increased VO_2 (Benoit et al., 1997). In this study we investigated the acute effects on gross efficiency (which is a function of both VO_2 and VCO_2) during hypoxic exercise . Interestingly, the SL group (most resistant to hypoxic exposure) had a significant reduction in gross efficiency whereas it remained unchanged in the LL group (Fig. 14). The figure reveal that the resulting metabolic efficiency was identical between the groups in hypoxia.
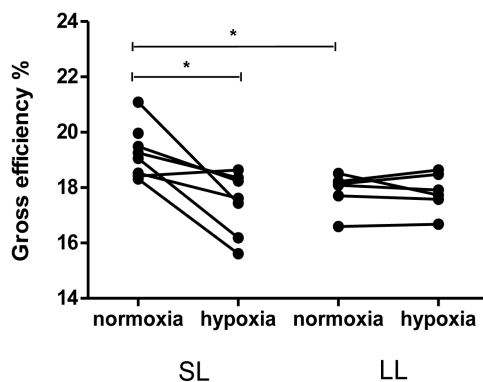
Figure 14. Gross efficiency during cycling exercise (100W) acutely changed in hypoxia for the group better able to maintain performance (small loss, SL) whereas no change was observed in the large loss group (LL). *P < 0.05.

Mitochondrial determinants of metabolic efficiency

In isolated mitochondria from the subjects, intrinsic respiration was lower in the SL group, evident by lower state 4 and state 3 respiration through both complex I and II and also during maximal ETS capacity (uncoupled respiration) (Fig 15A-F.) In order to examine if these effects were due to reduced mitochondrial density or reduced COX activity we analyzed protein abundance of several mitochondrial proteins by Western blotting and measured COX activity. We did not find any significant differences between the SL and LL group in the measured mitochondrial proteins or enzymatic activities.
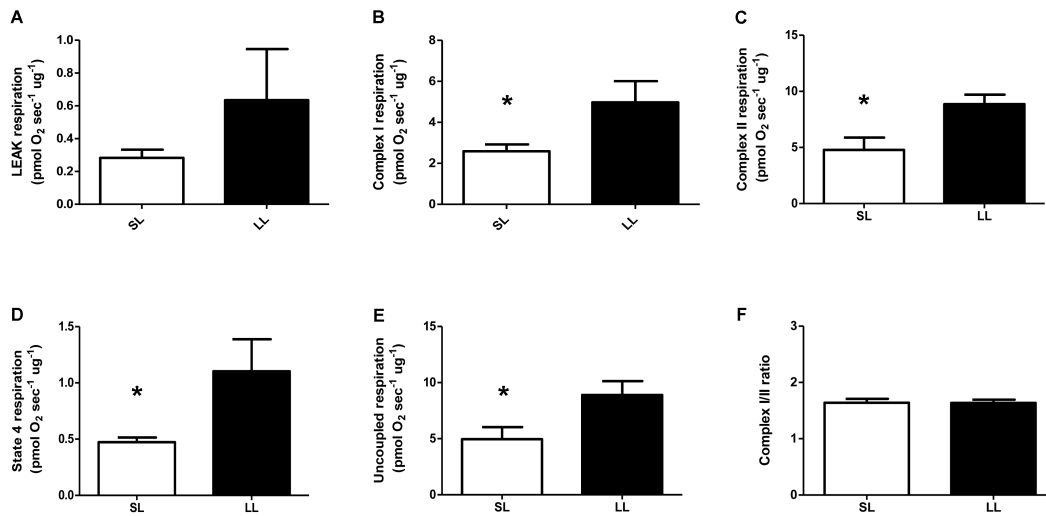
Figure 15. Lower intrinsic mitochondrial respiration was observed in subjects better able to maintain exercise capacity in hypoxia (SL) (A-F). (A) Leak respiration with complex I substrates without adentylates present. (B) State 3 respiration with pyruvate and malate (complex 1). (C) State 3 respiration with pyruvate, malate and succinate as substrates (complex I + II). (D) State 4 respiration (E) FCCP uncoupled respiration (F) State 3 complex I to complex II respiration ratio. *P < 0.05.

Oxygen tension dependent mitochondrial efficiency

P/O ratio is normally measured during ambient oxygen tension that poorly reflects the *in vivo* situation. We used a novel technique with the possibility to maintain a steady state oxygen tension during the measurements. We chose 0.3kPa O_2 since this oxygen tension has previously been shown to better reflect the intracellular oxygen tension during exercise (Richardson et al., 2001). P/O ratio was significantly increased at 0.3 kPa O_2 compared to 10 kPa, demonstrating that isolated mitochondria become more efficient in hypoxia (Fig. 16).
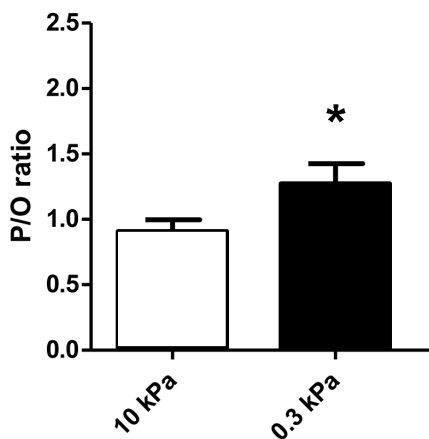
Figure 16. P/O ratio in isoloated mitochondria during steady state oxygen tensions (10 and 0.3kPa). *P < 0.05

This study demonstrates that a high ME during normoxia is crucial to maintain performance in hypoxia. Moreover, the SL group (showing high ME in normoxia) were able to acutely decrease their ME during hypoxia, thereby trading efficiency for power output, supporting that the bioenergetic efficiency may be dynamically regulated and optimized according to the maximum power principle. The LL group are probably already optimized for maximal power output and therefore unable to adapt in the same manner as the SL group. Interestingly, high altitude natives have previously been shown to have a high ME (Curran et al., 1998; Hochachka et al., 1991). ME did not correlate with any qualitative variable in mitochondria. Instead, intrinsic mitochondrial respiration was lower in the SL compared to the LL group. Isolated mitochondria operate in the low-efficiency range where an increased efficiency is expected to yield a higher power output. Indeed, acute decrease of oxygen tension from ~10 kPa to 0.3 kPa increased the P/O ratio and the mechanistic explanation could be a reduced proton slip at cytochrome c oxidase, previously shown during COX inhibition by nitric oxide and cyanide (Clerc et al., 2007). Our study does not reveal the mechanistic explanation for the acute decrease in ME during hypoxic exercise. Mitochondrial complexes have recently been shown to organize into supercomplexes (Lapuente-Brun et al., 2013) and this may be a route for self-organizing and favouring an efficiency optimal to maximize power output according to external stimuli (Karsenti, 2008). These findings show that instead of incresing efficiency, the body rather favours to adjust the efficiency to what corresponds to maximum power production depending on the metabolic demands.

Paper VI

Control of Energy Expenditure by Cytochrome C Oxidase Subunit IV-2

The pronounced variation in RMR among subjects with similar phenotypes is still partly unexplained (Johnstone et al., 2005) but differences in mitochondrial efficiency may be a contributing factor. At high membrane potential and high ATP/ADP ratio, proton slip occurs at COX (Frank and Kadenbach, 1996; Kadenbach, 2003) and this process may be affected by the COX subunit isoform composition. COX subunit IV exists in two isoforms: COX IV-1 is ubiquitously expressed in mammalian tissues while COX IV-2 has previously only been shown in adult lung tissue, fetal lung and muscle (Hüttemann et al., 2001) and in neurons (Horvat et al., 2006; Misiak et al., 2010; Singh et al., 2010). In this paper we wanted to investigate whether the COX IV-2 isoform is present in human skeletal muscle and if the composition of isoforms affects resting metabolic rate. Sixteen healthy volunteers were recruited and subjected to measurements of RMR and extraction of skeletal muscle biopsies.

COX IV-2 in human skeletal muscle and correlation to RMR

We found that COX IV-2 was expressed in normal human skeletal muscle (Fig. 17). In addition, the ratio of COX IV-2/COX IV-1 showed significant inverse correlation with resting metabolic rate (fig. 18) indicating that COX IV-2 may contribute to the regulation of RMR.
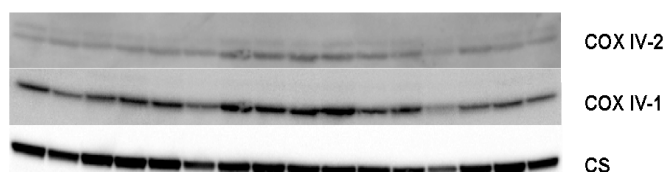

Figure17. Representative picture of immunoblot analysis of COX IV subunits in skeletal muscle. CS=citrate synthase
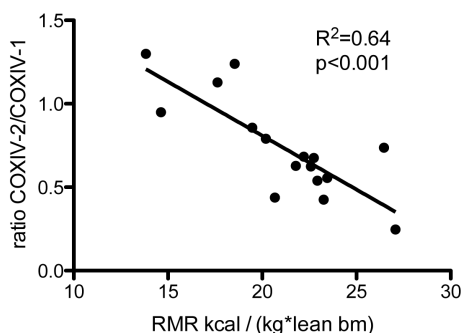
Figure 18. Correlation between skeletal muscle COX IV-2/COX IV-1 ratio and resting metabolic rate.

Effects of concurrent overexpression of COX IV-2 and knockdown of COX IV-1 on cellular respiration

Primary myoblasts were isolated and cultured from the human biopsies. Following differentiation, COX IV-2 was overexpressed and COX IV-1 was knocked down. Cellular respiration was then analysed with high resolution respirometry. In line with the influence of high COX IV-2/COX IV-1 ratio on RMR, this transfection lowered the basal and leak cellular respiration by 66% and 54%, respectively (Fig. 19A, B). We measured overall COX activity to exclude that a generalised impairment of COX in the treated cells was the reason for the observed effect and it was unaffected.
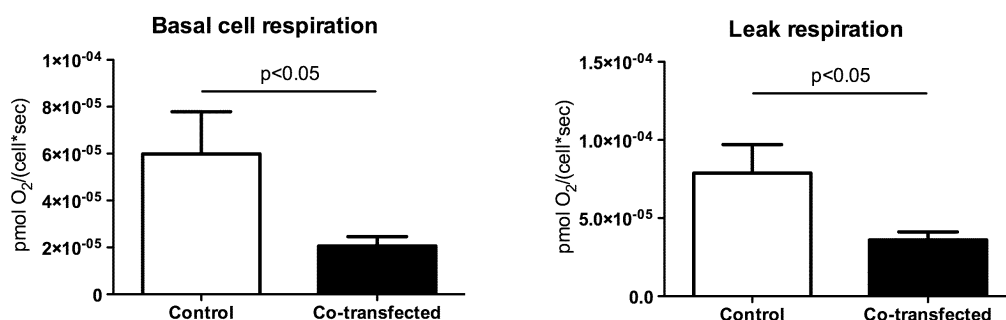
Figure 19. Comparison of respiration in cells overexpressing COX IV-2 and concomitant knockdown of COX IV with control cells. (A) Basal cell respiration. (B) Leak respiration in permeabilized cells.

Effects of concurrent overexpression of COX IV-2 and knockdown of COX IV-1 on ROS production

In order to investigate the influence of COX subunit IV isoform composition on ROS production, hydrogen peroxide (H_2O_2) generation was measured spectrophotometrically (Amplex ultrared) during ongoing respirometry. H_2O_2 production during basal respiration (Fig. 20) as well as leak and ADP stimulated respiration was significantly reduced in the treated cells compared to control cells.
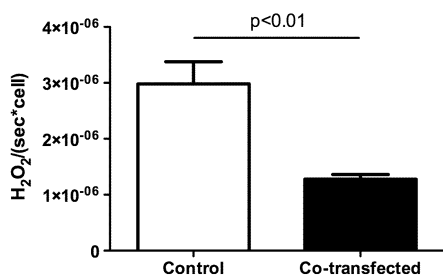
Figure 20. H_2O_2 production during basal cell respiration in control cells and cells overexpressing COX IV-2 with concomitant knockdown of COX IV-1.

This study shows that COX IV-2 protein is present in normal human skeletal muscle and that this subunit isoform could partly explain inter-individual variations in RMR. The profound reduction in basal cell respiration following concurrent overexpression of COX IV-2 and knockdown of COX IV-1, without affecting the overall COX activity, further strengthens this suggestion. This is partly supported by studies showing that COX IV-2 improves mitochondrial efficiency during hypoxia (Fukuda et al., 2007). It is not known how a COX isoform switch may affect COX H^+/e^- stoichiometry. The lower basal cell respiration may depend on COX IV-2 mediated reduction in COX proton slip, known to be present with COX IV-1 (Murphy, 1989). Another explanation could be COX inhibition by NO that is known to increase mitochondrial efficiency (Antunes et al., 2007; Clerc et al., 2007). The mechanism would be enhanced nitrite reduction to NO by COX IV-2 which is supported by findings in yeast where nitrite reduction was more efficient when the COX IV-2 analogue Vb was present (Castello et al., 2008). This could also be a possible explanation for the reduced RMR observed in subjects with a higher COX IV-2/ COX IV-1 ratio.

We found lower levels of H_2O_2 in the myotubes overexpressing COX-IV-2. Fukuda et al. have previously shown lower ROS generation during hypoxia in cells overexpressing COX-IV-2. In contrast Singh et al. found increased ROS generation in isolated mitochondria extracted from astrocytes expressing COX IV-2 (Singh et al., 2009). Since we were only able to detect H_2O_2 escaping the cells in our set up, the explanation for our findings may consist of an upregulation of endogenous antioxidants and/or downregulation of cellular ROS generating enzymes such as monoamine oxidases (MAOs) or NADPH oxidases (NOXs). Together, our data implicates COX IV-2 in the control of energy expenditure and ROS homeostasis in humans and may have implications in pathologies and weight control.

CONCLUSIONS

- Dietary nitrate improves mitochondrial efficiency and downregulates proteins involved in uncoupling of oxidative phosphorylation

- Dietary nitrate increases contractile force in fast twitch muscle in mice, at frequencies corresponding to normal movement *in vivo*. This is coupled to an increase in myoplasmic free $[Ca2^+]$ and increased expression of the Ca^{2+} handling proteins DHPR and CASQ1.

- Dietary nitrate impairs static apnea performance by accelerating arterial oxygen desaturation. This probably occurs through NO-mediated attenuation of the diving response.

- Dietary nitrate reduces basal metabolic rate by ~ 4% without affecting thyroid hormone levels.

- Acute administration of nitrite reduces basal respiration in differentiated primary myotubes

- Individuals with high metabolic efficiency in normoxia better maintain exercise performance in hypoxia compared with individuals with a low metabolic efficiency in normoxia

- Individuals with high metabolic efficiency acutely decrease their metabolic efficiency in hypoxia thereby trading efficiency for maintained power output. This supports an optimization of bioenergetic efficiency according to the maximum power principle.

- Individuals with higher metabolic efficiency in normoxia have lower intrinsic mitochondrial respiration.

- The cytochrome c oxidase subunit IV isoform 2 is present in human skeletal muscle.

- Skeletal muscle COX IV-2/COX IV-1 ratio negatively correlates with resting metabolic rate in humans.

- Concurrent overexpression of COX IV-2 and knockdown of COX IV-1 attenuates basal cell respiration and ROS generation in differentiated human primary myotubules.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Since the discovery of the mammalian nitrate-nitrite-NO pathway in 1994 the physiological, therapeutical and nutritional aspects of this alternative system for NO generation has attracted substantial scientific interest. Numerous studies have now shown beneficial effects of nitrate and nitrite on blood pressure (Larsen et al., 2006; Webb et al., 2008) , ischemia-reperfusion injury (Duranski et al., 2005; Shiva et al., 2007), inflammation (Jädert et al., 2012) and exercise performance (Jones, 2014). In this thesis we focused on the effects of dietary nitrate on mitochondrial and muscle function related to exercise as well as effects on resting metabolic rate. In addition, we have investigated how metabolic efficiency predicts tolerance to hypoxia and how mitochondrial COX subunit composition affects bioenergetics in humans. Importantly, the dosing of nitrate in all studies in this thesis (humans and mice) was in the range that can easily be achieved by a vegetable rich diet, which brings interesting nutritional aspects to our findings. The fact that a relatively short-term dietary regimen can influence expression of important mitochondrial proteins and affect whole body energetics during both rest and exercise is intriguing. There are several implications of these findings.

The nutritional aspects are obvious since nitrate is abundant in vegetables such as spinach, ruccola, lettuce and beetroot. The sports community early adopted beetroot juice to enhance performance and there are currently numerous top athletes that consume beetroot juice in hope to perform better. Within the scientific community there are presently more than 50 studies on different aspects of inorganic nitrate on exercise performance, with a majority showing reduced oxygen cost and increased performance. In most of these studies beetroot juice has been used as a natural source of inorganic nitrate. It seems clear that it is nitrate that is responsible for the effects since beetroot juice slectively depleted in nitare is inactive (Kelly et al., 2014). Some studies indicate that elite athletes respond less to nitrate which may be due to their overall better NO status (higher plasma nitrate and nitrite) compared to less trained individuals.
Even if not studied in this thesis it is worth mentioning that the majority of studies within the nitrate-nitrite-NO field are related to the cardiovascular system. From a nutritional viewpoint this is interesting since a diet rich in vegetables is protective against cardiovascular disease and in several studies green leafy vegetables stand out as particularly beneficial (Carter et al., 2010; Cooper et al., 2012; Hung et al., 2004). However, the active substance(s) has not yet been pinpointed and ongoing studies are now investigating if nitrate contributes to the beneficial effects of a green diet. If that would be the case we need to discuss if the current negative view on dietary nitrate in our society is justified and whether the existing strict regulations of nitrate concentrations in food and drinking water should be maintained.

The physiological role of endogenously generated nitrate and nitrite is less clear. Exogenous nitrate or nitrite is most often used albeit at low doses as in this thesis. Several *in vitro* studies show effects at concentrations similar to those circulating *in vivo* (Gao et al., 2014). Since there is no apparent way, either by a pharmacological inhibitor or by gene deletion to inhibit systemic nitrite reduction, it is difficult to design proper experiments. However, nitrate requires reduction to nitrite by oral bacteria in order to be effective and therefore temporary inhibition of oral bacteria by an antisepetic mouthwash (Hendgen-Cotta et al., 2012) or the use of germfree mice is of value. Indeed, it has been shown that endogenously generated nitrate from NO synthases is recycled to bioactive NO and is sufficient to participate in regulation of

blood pressure and concomitant increase in circulating nitrite in humans. This was shown by a rise in blood pressure after administration of an oral antiseptic mouthwash to fasting healthy humans (Kapil et al., 2013). Whether the endogenous levels of nitrate are enough also to participate in regulation of basal metabolic rate and oxygen consumption during exercise has not yet been studied.

The therapeutic implications of the present findings may go beyond improving exercise performance. The nitrate-induced improvement in mitochondrial efficiency could be of importance in metabolic and cardiovascular diseases in which dysfunctional mitochondria play a central role. The changes in calcium handling and muscular function by dietary nitrate indicate a potential therapeutic role for nitrate. For example, impaired calcium handling in skeletal muscle has recently been reported in mitochondrial myopathy (Aydin et al., 2009). Thus, nitrate supplementation may improve contractile function in diseases with muscle weakness involving impaired calcium handling.

The effect of dietary nitrate on resting metabolic rate is interesting and it is tempting to speculate on implications for weight control and perhaps even for longevity. Caloric restriction has similar effects on resting metabolic rate as nitrate and increases life span in a number of species(Heilbronn and Ravussin, 2003). There are two theories trying to explain survival in terms of mitochondrial respiration and ultimately basal metabolic rate. The *rate of living* theory (Harman, 1956; Speakman, 2005; Speakman et al., 2004) proposes that a continuous ROS production during normal respiration eventually leadsto the oxidation of mitochondrial DNA, proteins and lipids. Attempts have been made to link maximal survival to oxygen utilization per unit weight that resulted in modest support for this theory (Speakman, 2005). On the other hand, the *uncoupling to survive* theory assumes that rather than a fixed proportion of consumed oxygen being diverted to ROS generation, uncoupling is important to minimize mitochondrial ROS production (Speakman et al., 2004). Whether the effects of dietary nitrate on basal metabolic rate and other variables may have implications on life span is an open question and indeed an interesting subject for future investigations.

In conclusion, mitochondrial function is governed by numerous independent factors of which we have studied a few in this thesis. We have shown that dietary nitrate can improve mitochondrial and muscular efficiency with concomitant reduction in oxygen consumption during rest and exercise. In addition, we suggest that metabolic efficiency predicts exercise tolerance during hypoxia and that metabolic efficiency adapts during exercise to achieve optimal power output. Finally, we have shown that resting metabolic rate is related to mitochondrial subunit composition. Together, these studies create a platform for further investigations centred around dietary nitrate, mitochondrial function and metabolism.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I denna avhandling har vi studerat hur ämnet nitrat (NO_3^-), som förekommer rikligt i vår kost, främst i gröna grönsaker, kan påverka energiomsättningen hos människa i vila och under arbete. Vi har identifierat mitokondrierna som ett mål för effekterna av nitrat och i avhandlingens senare del har vi även studerat hur vissa faktorer i mitokondriernas funktion påverkar vår energiomsättning.

Mitokondrierna är cellernas kraftverk som tillverkar ämnet adenosin trifosfat (ATP), vilket är energivalutan i kroppens processer. Vid nedbrytning av socker och fett tillförs elektroner till mitokondrierna, där elektronerna sedan vandrar i den så kallade andningskedjan. När elektronerna transporteras i andningskedjan pumpas positivt laddade vätejoner (protoner) över ett inre membran i mitokondrien och det byggs upp en elektrokemisk gradient som sen används för att skapa ATP. För att detta skall fungera måste mitokondrien ha tillgång till syre (O_2) som tar emot elektronerna i slutet av andningskedjan. Syre är den slutliga elektronacceptorn och reduceras då till vatten. Således behövs både näring från maten för leverans av elektroner och syre från luften via andningen för produktion av ATP. Trots att mitokondrien är sinnrik i sin mekanism så har den som alla system en begränsad verkningsgrad. Således är transporten av vätejoner över det inre mitokondriemembranet inte perfekt och man talar om ett protonläckage eller frikoppling (uncoupling) i systemet. Vätejonerna läcker tillbaka över membranet utan att bilda ATP. Denna frikoppling bildar värme istället för ATP och bidrar delvis till vår kroppstemperatur. En annan process som bidrar till en mindre verkningsgrad är då vissa syremolekyler reduceras till den fria radikalen superoxid (O_2^-) istället för vatten. Hög produktion av superoxid kan vara farlig och bidra till sjukdom men normal bildning av superoxid anses viktig och behövs för signalering i cellerna.

Den lilla gasmolekylen kvävemonoxid (NO) bildas kontinuerligt i kroppen med hjälp av specifika enzym, så kallade NO-syntaser. Det är känt sedan lång tid att NO har stor betydelse för en rad viktiga funktioner i kroppen såsom reglering av blodflöde och blodtryck, immunförsvaret samt även reglering av mitokondriefunktion. Minskad NO bildning anses bidra till åderförkalkning vid normalt åldrande samt ha patologisk betydelse vid hjärtkärlsjukdomar. För 20 år sen upptäckte vår forskargrupp att NO även kan bildas från de oorganiska anjonerna nitrat (NO_3^-) och nitrit (NO_2^-) utan medverkan av NO-syntaser. Dessa joner är restprodukter av NO men finns också i vår kost. Nitrat förekommer rikligt i gröna grönsaker och när man äter dessa kommer NO att genereras i kroppen. Detta sker genom så kallad reduktion och för att detta skall ske behövs både bakterier i munhålan samt specifika enzymer i kroppen. Efter intag av nitratinnehållande grönsaker tas nitratet upp i magtarmkanalen. En del av det nitrat som cirkulerar i blodbanan kommer sedan att tas upp i våra spottkörtlar och utsöndras i saliven. Den normala bakteriefloran i munnen kommer där att reducera nitrat till nitrit (NO_2^-) som sedan sväljs och nitrit tas upp i blodbanan. I kroppen finns en rad olika enzym som sedan kan reducera nitrit vidare till aktiv NO. Detta nitrat-nitrit-NO system är intressant av flera aspekter. Dels så verkar det under andra betingelser än de klassiska NO-syntaserna och dels så kan man via dieten fylla på NO nivåerna i kroppen genom intag av nitratinnehållande grönsaker.

I det första delarbetet **(Paper I)** ville vi undersöka om intag av nitrat kunde förbättra mitokondriernas funktion och på så sätt minska syrebehovet vid fysiskt arbete. Denna frågeställning byggde på tidigare kunskap från vår grupp där vi visat att nitrat kan minska syrebehovet vid fysiskt arbete men mekanismen var fortfarande okänd. Friska försökspersoner undersöktes vid 2 tillfällen; efter 3 dagars intag av nitrat (motsvarande

mängden i 200-300 gram spenat eller 2-3 rödbetor) eller efter intag av koksalt (placebo). Vi tog vävnadsprover från lårmuskel ur vilka mitokondrier isolerades som sedan analyserades i en oxygraf, där mitokondriernas funktion kunde mätas. Försökspersonerna fick också utföra ett cykelarbete och resultatet från detta jämfördes med mitokondriernas funktion.

Vi kunde konstatera att nitratladdningen hade gjort mitokondrierna mera effektiva gällande produktion av ATP. Mindre syre krävdes per bildad ATP molekyl. Vi fann ett starkt samband mellan förbättringen i mitokondriefunktion och det minskade syrebehovet under cykelarbete, vilket talar för att förbättringen på helkroppsnivå efter nitrat beror på en förbättrad effektivitet i mitokondrierna. Våra mätningar i oxygrafen tydde på ett minskat protonläckage efter nitratintag som förklaring till den ökade effektiviteten och vi fann också tecken på att ett par proteiner som är inblandade i mitokondriellt protonläckage (ANT och UCP-3) hade nedreglerats. Slutsatserna i denna studie är att den lägre syrekonsumptionen under arbete efter intag av nitrat kan förklaras av ökad mitokondriell effektivitet. Effektivitetsökningen i sin tur verkar bero på ett lägre protonläckage och en lägre halt av proteiner involverade i denna mekanism.

I andra delarbetet (**Paper II**) ville vi undersöka om nitrat även kan ha effekter på muskelfunktion. I möss undersökte vi effekterna av dietärt nitrat på muskelkraft (kontraktilitet) och kalciumhantering. Möss fick nitrat i dricksvattnet under 7 dagar och jämfördes med en kontrollgrupp. Muskelprover stimulerades elektriskt med olika frekvenser för mätning av kontraktilitet.

Det visade sig då att de nitratladdade mössens snabba muskler (typ II) hade en mera uttalad kraftutveckling jämfört med kontrollmössen. En muskel kontraherar som en följd av kalciumfrisättning från depåer (sarkoplasmatiska retiklet, SR) i cellen. Vi mätte kalciumkoncentrationerna under elektrisk stimulering och såg en ökad kaciumfrisättning i mössen som hade druckit nitrat. Vi fann också att lagringsförmågan av kalcium i SR hade ökat samt att proteinet CASQ1, som binder stora mängder kalcium, hade uppreglerats. Även mängden av DHPR, ett annat protein som är involverat i kalciumfrisättning, hade ökat efter nitratintag. Våra fynd har senare bekräftats i human muskel där man såg att 7 dagars intag av nitrat ökade kraftutvecklingen med 8% vid elektrisk stimulering. Slutsatserna i vår studie är att dietärt nitrat bidrar till ökad kontraktilitet i snabba muskelfibrer. Förklaringen till denna förändring är ett ökat uttryck av proteinerna CASQ1 och DHPR som dels ökar lagringskapaciteten av kalcium i SR och bidrar till ökad frisättning av kalcium vid nervsignalering. Dietärt nitrat har därmed potentialen att visa sig lindra tillstånd där man ser nedsatt muskelfunktion till följd av försämringar i kalciumhanteringen.

Omvandlingen av nitrit till NO i kroppen är potentierad vid låg syrehalt (hypoxi) och lågt pH. Vi ville därför undersöka effekterna av dietärt nitrat på syrekonsumtion i en human modell av generell hypoxi. Hypotesen var att nitrat skulle minska syrebehovet vid långvarig andhållning och därmed öka andhållningstiden. I delarbete tre (**Paper III**) rekryterades erfarna fridykare som kan hålla andan länge (>4 min), vilket leder till låg syremättnad i blodet. Försökspersonerna undersöktes vid två tillfällen (med och utan nitrat) på samma sätt som i delarbete I. De fick hålla andan i vila (2 min, 4 min samt maximal andhållning) och vid cykling (60, 90 sek och maximal andhållning).

I motsats till vår hypotes så konsumerades det mer syre i vila när försökspersonerna hade intagit nitrat i jämförelse med placebo. Detta visade sig genom signifikant lägre arteriell syresaturation efter 4 min och maximal andhållning. Maximala andhållningstiden var signifikant kortare efter nitratsupplementering. Dietärt nitrat hade ingen signifikant effekt på syreförbrukningen under andhållning vid arbete. Vid andhållning triggas den så kallade dykresponsen vilken består av kärlsammandragning

och minskad hjärtfrekvens och anses minska syrebehovet. Våra data tyder på att nitrat hämmade dykresponsen vilket skulle kunna förklara varför syrekonsumptionen var högre efter nitratsupplementeringen. Sammanfattningsvis så visade denna studie att dietärt nitrat ökar syreförbrukningen vid långvarig andhållning i vila.

Den ökade mitokondriella effektiviteten efter nitratintag som vi såg i första delarbetet skulle kunna ha effekter på vilometabolismen. I fjärde delarbetet **(Paper IV)** studerade vi vilometabolismen hos friska försökspersoner med ett liknande protokoll som i delarbete 1. Vilometabolismen mättes med indirekt kalorimetri och beräknades från syrekonsumtion och koldioxidproduktion.
Intag av nitrat sänkte syrekonsumtionen med ca 5% och vilometabolismen med ca 4%. Vi såg ingen förändring tydande på en förskjutning av utnyttjandet av substrat från fett mot glukos. Vi fann ett starkt samband mellan förändringen av salivnitrat mot förändringen av vilometabolismen vilket indikerar att upptag av nitrat i spottkörtlarna och utsöndring i saliv är viktigt för dessa effekter. Vi mätte även syreförbrukningen i isolerade muskelceller från försökspersonernas lårmuskel. Tillsatts av nitrit sänkte respirationen med 40%. Sammanfattningsvis så visade studien att vilometabolismen sänks efter nitratsupplementering. Detta skulle kunna ha konsekvenser för viktkontroll men kanske även på livslängd, då exempelvis kalorirestriktion som har liknande effekter på vilometabolismen, har visat sig öka livslängden i en rad olika däggdjur.

Enligt principen "Maximum power" föreslås att biologiska system tenderar att organisera sig mot en effektivitetsnivå som ger maximal produktion av kraft. I delarbete V **(Paper V)** ville vi på människa undersöka om en snabb anpassning i metabol effektivitet kan ske för att bibehålla arbetsförmågan vid hypoxi. För detta ändamål cyklade friska försökspersoner varpå metabol effektivitet (användbar mekanisk energi under cyklingen i förhållande till den totala energiomsättningen) uppmättes i normoxi (21% O_2) respektive hypoxi (16% O_2). Efter att ett maxtest genomfördes i normoxi respektive hypoxi så delades individerna upp i två grupper där den ena förlorade mycket arbetskapacitet (Large Loss, LL) medan den andra var mera tolerant mot hypoxin och förlorade mindre arbetskapacitet (Small Loss, SL). Intressant nog hade SL gruppen signifikant högre metabol effektivitet i normoxi än LL gruppen. När vi sedan mätte metabola effektiviteten under hypoxi så hade SL gruppen minskat sin effektivitet i förhållande till normoxi medan LL gruppen hade samma effektivitet som under normoxi. Det verkar som SL gruppen sänkte sin metabola effektivitet under hypoxi för att uppnå optimal kraftutveckling (maximal power). Mekanismen för denna akuta anpassning är oklar och kan innefatta hypoxiinducerad mitokondriell frikoppling eller proteinmodifieringar som bidrar till sänkt effektivitet. Sammanfattningsvis så visade denna studie att personer som hade en högre effektivitet när de cyklade i normoxi bättre kunde bibehålla sin kapacitet vid hypoxi. Detta skedde under samtidig effektivitetsförsämring på helkroppsnivå vilket kan vara en förklaring på att de bibehöll prestationen i hypoxi.

Trots identisk vikt, fettfri massa, kön och ålder så kan vilometabolismen variera kraftigt mellan olika individer. En stor del av den interindividuella variationen är fortfarande oförklarad men eftersom produktionen av ATP är kopplad till syrekonsumption så skulle man kunna tänka sig att skillnader i mitokondriell effektivitet skulle kunna påverka vilometabolismen. Det är känt att cytokrom c oxidas (det sista enzymet i mitokondriens andningskedja) kan vara mer eller mindre effektivt under olika omständigheter. Detta enzym består av 14 subenheter där subenhet 4 starkt bidrar till att aktiviteten av enzymet kan regleras beroende tillgång till energi. Denna subenhet finns i 2 olika isoformer. I sjätte delarbetet **(Paper VI)** undersökte vi om subenhet 4 isoform 2

(COX IV-2) finns i human skelettmuskel och om nivån av denna i olika individer skulle kunna förklara skillnader i vilometabolism. Vi rekryterade friska män där vi mätte vilometabolism, tog skelettmuskelbiopsier och mätte nivåerna av dessa subenheter. Vi isolerade även satellitceller från muskelbiopsierna och studerade hur överuttryck respektive "knock-down" av dessa isoformer påverkade respirationen.

Vi upptäckte dels att COX IV-2 finns uttryckt i human skelettmuskel och att kvoten av COX IV-2/COX IV-1 korrelerade starkt med vilometabolismen vilket indikerar att kompositionen av dessa isoformer kan vara viktigt för vilometabolismen. När vi i muskelcellerna överuttryckte COX IV-2 under samtidig "knock-down" av COX IV-1 minskade den basala respirationen med över 60%, vilket stödjer fynden med minskat syrekrav på helkroppsnivå i individerna som har högre nivåer av COX IV-2.

Sammantaget så visar denna studie att sammansättningen av isoformerna för subenhet IV (COX IV-2/COX IV-1) påverkar vilometabolismen. Detta styrktes av att överuttryck och "knock-down" av COX IV-2 respektive COX IV-1 bidrog till lägre cellrespiration.

Sammanfattning

Denna avhandling visar att dietärt nitrat, i mängder som kan uppnås genom en grönsaksrik diet, har effekt på en rad funktioner kopplade till metabolism, mitokondriefunktion och muskelkraft. Vi har visat att dietärt nitrat sänker syreförbrukningen i vila och under arbete. Detta sker genom att öka mitokondriernas effektivitet samt sannolikt också genom ökad muskelstyrka. Vi har också visat att den metabola effektiviteten verkar avgörande för tolerans vid arbete under syrebrist och kan därmed vara avgörande vid sjukdomstillstånd tillstånd med låg syreleverans till vävnaderna såsom vid perifer cirkulationsinsufficiens. De stora variationerna i vilometabolism mellan individer verkar delvis kunna förklaras av sammansättningen av cytokrom c oxidas subenheter, där isoformerna av subenhet IV verkar spela stor roll.

Sammantaget visar våra data på intressanta terapeutiska och näringsfysiologiska aspekter av oorganiskt nitrat. Framtida studier kommer att avslöja om dessa fynd kan omsättas i nya terapier eller kostråd baserat på kunskapen om nitrats fysiologiska effekter.

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