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NITRIC OXIDE FORMATION FROM INORGANIC NITRATE AND NITRITE

Contribution from eukaryotic and
prokaryotic pathways

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To my beloved family

“Let your food be your medicine and your medicine be your food.”
Hippocrates (460–359 BC)

ABSTRACT

Nitric oxide (NO) is an essential signaling molecule that plays a central role in a broad range of physiological functions. Classically, NO is synthesized from L-arginine and molecular oxygen by NO synthases. Once formed, it is rapidly oxidized to nitrite and nitrate. These two inorganic anions were previously considered to be inert end products but this view is now being seriously challenged by research revealing that nitrite can be physiological reduced to again generate NO. The reduction of nitrite *in vivo* seems particularly enhanced during hypoxia and acidosis; conditions when the oxygen-dependent NO-synthase pathway is dysfunctional. Besides the endogenous formation of nitrate and nitrite by NO synthase, these anions are also ingested naturally via the diet. The first step in bioactivation of nitrate is formation of the more reactive nitrite anion; a reaction suggested to involve oral nitrate reducing bacteria. It has been generally viewed that mammalian cells cannot metabolize the stable nitrate anion.

In the present thesis, we intended to further characterize NO generation from the nitrate-nitrite-NO pathway. In particular we have studied the importance of commensal bacteria in nitrate metabolism and attempted to explore if mammalian tissues are also capable of nitrate reduction. We also studied possible interactions between the classical NO synthase pathway and the nitrate-nitrite-NO pathway.

We show that bacteria in the gastrointestinal tract play an interesting role in mammalian NO biology. Besides the bioactivation of nitrate in the oral cavity to form nitrite, bacteria in the small and large intestine can catalyze the same reaction and also the subsequent reduction of nitrite to form NO. NO formation in the gut can be stimulated *in vivo* by supplementation with dietary nitrate and probiotic bacteria.

In further studies involving also germ-free mice, we surprisingly find that inorganic nitrate is enzymatically reduced to nitrite in tissues and we identify the enzyme xanthine oxidoreductase (XOR) as the dominant nitrate reductase. Mammalian nitrate reductase activity is enhanced during hypoxic conditions but is also active during normoxia. The functional consequences of this nitrate reductase activity were studied after nitrate administration *in vivo*. Nitrate attenuated the increased blood pressure caused by an NO synthase inhibitor and prevented the severe decline in blood flow during post-ischemic reperfusion.

The expression of XOR is enhanced in tissues of germ free mice, which may reflect a feedback response to the absence of bacterial nitrate reduction in these animals. Such crosstalk is further supported in a study of long-term dietary nitrate supplementation in rats, in which expression of phosphorylated eNOS in aortic tissue and eNOS activity was down-regulated after nitrate supplementation. All together these data suggest a crosstalk between NOS-independent and NOS-dependent pathways in control of NO vascular homeostasis.

In summary, the present thesis helps to draw a new picture of mammalian NO generation which occurs by serial reductions of the supposedly inert anions nitrate and nitrite. In this pathway both eukaryotic and prokaryotic pathways contribute to formation of NO and maintenance of homeostasis. Intriguingly, NO formation from nitrate in the gastrointestinal tract, the cardiovascular system and elsewhere, can be controlled by simple dietary interventions with resulting physiological effects.

LIST OF PUBLICATIONS

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

I. Tanja Sobko, Liyue Huang, Tore Midtvedt, Elisabeth Norin, Lars E. Gustafsson, Mikael Norman, Emmelie Å. Jansson, Jon O. Lundberg. (2006) Generation of NO by probiotic bacteria in the gastrointestinal tract. *Free Radic Biol Med.* Sep; 15;41(6):985-91

II. Emmelie Å Jansson, Liyue Huang, Ronny Malkey, Mirco Govoni, Carina Nihlén, Annika Olsson, Margareta Stensdotter, Joel Petersson, Lena Holm, Eddie Weitzberg, Jon O. Lundberg. (2008) A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nature Chemical Biology*. Jul; 4(7):411-7

III. Liyue Huang, Sara Borniquel, Jon O. Lundberg. (2010) Enhanced xanthine oxidoreductase expression and tissue nitrate reduction in germ free mice. *Nitric Oxide*. Feb; 15;22(2):191-5

IV. Liyue Huang, Mattias Carlström, Sara Borniquel, Eddie Weitzberg, Jon O. Lundberg. Evidence of a crosstalk between NOS independent and NOS dependent NO pathways in control of vascular NO homeostasis. *Manuscript*

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LIST OF ABBREVIATIONS

CFU	colony-forming unit
cGMP	cyclic guanosine monophosphate
Conv	conventional
Deoxy-Hb	deoxyhemoglobin
eNOS	endothelial nitric oxide synthase
GF	germ free
GI	gastrointestinal
GTP	guanosine triphosphate
HCl	hydrochloric acid
HNO_2	nitrous acid
HPLC	high performance liquid chromatography
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MAP	mean arterial pressure
met-Hb	methemoglobin
N_2	dinitrogen
N_2O_3	dinitrogen trioxide
NADPH	nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
O_2^-	superoxide
nNOS	neuronal nitric oxide synthase
$ONOO^-$	peroxynitrite
oxy-Hb	oxyhemoglobin
PKG	protein kinase G
ppb	parts per billion
ppm	parts per million
RNI	reactive nitrogen intermediate
sGC	soluble guanylyl cyclase
SNO	S-nitrosothiol
XOR	xanthine oxidoreductase

1 INTRODUCTION

Nitrogen is essential for any life. It is a component in all amino acids and is present in the bases that constitute nucleic acids, such as DNA and RNA. The by far dominating source of nitrogen in nature is the atmosphere, where it exists mainly as nitrogen gas (N_2). However, N_2 is extremely inert and must be fixed and interconverted to other forms of nitrogen before it can be utilized by plants and animals. This occurs in a fundamental process known as the nitrogen cycle[1,2]. In this cycle, which is mainly orchestrated by bacteria, nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO) are essential intermediates. NO is of particular interest in mammalian biology since it represents a potent signaling molecule[3,4]. While bacteria can generate NO anaerobically via reduction of higher nitrogen oxides, mammals have instead developed an oxidative pathway for the generation of this gas. This occurs via oxidation of the amino acid L-arginine, in a process governed by specific enzymes - the NO synthases (NOSs). Recent lines of research suggest that formation of NO in mammals might actually stem from both reductive- and oxidative pathways[5-7]. The present thesis explores mechanisms of NO generation in mammals and how prokaryotic and eukaryotic cells in our bodies interact to produce this potent biological messenger.

Nitrate and nitrite have been in use for long to preserve food and even in medical care. Potassium nitrate (saltpetre) was used to treat heart diseases as early as in the medieval times according to a medical recipe discovered in a Buddhist grotto in Dunhuang, the crossroads of the ancient Southern Silk Route in China. Throughout history, several drugs that contain nitrogen molecules have been used: nitrous oxide (N_2O), so call laughing gas discovered in the 19th century, is used as a partial anesthetic. Nitroglycerine and nitroprusside are used as vasodilators, acting via release of nitric oxide to regulate blood pressure and heart conditions such as angina and chronic heart failure.

1.1 NITRIC OXIDE – FROM AIR POLLUTANT TO CELEBRATED BIOLOGICAL MESSENGER

Nitric oxide was discovered in 1772 by Joseph Priestley. He described it as a colorless and toxic gas which he referred to as "nitrous air". For over two hundred years NO received the label of being a toxic gas and unwanted air pollutant. NO is produced during combustion processes and is present in high concentration in car exhaust and cigarette smoke. The view of NO being solely a dreaded air pollutant ceased abruptly in the 1980s when several lines of research converged, ultimately culminating in the discovery of endogenous NO formation[8-11]. As it turned out, not only did our bodies produce NO themselves, but it was made for specific purposes. In fact, it was shown to be a central signaling molecule involved in essentially all important aspects of mammalian biology, including regulation of blood flow, peripheral nerve transmission, brain function, gut motility, penile erection, metabolism, immunity and more. In 1998 three American researchers were awarded the Nobel Prize in Physiology or Medicine for their discoveries of NO as a signaling molecule in the cardiovascular system.

Box 1. Some relevant biological reactions involving NO, nitrite and nitrate

NO generation from the L-Arginine-NO synthase pathway

$$L\text{-}Arginine + O_2 \rightarrow NO\bullet + L\text{-}Citrulline$$

NO formation from the nitrate-nitrite-NO pathway

Bacterial nitrate reductase

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$$

Nitrite reduction

Deoxyhaemoglobin/myoglobin

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow NO\bullet + HbFe^{3+} + OH^-$$

Xanthine oxidoreductase

$$NO_2^- + Mo^{4+} + H^+ \rightarrow NO\bullet + Mo^{5+} + OH^-$$

Protons

$$NO_2^- + H^+ \rightarrow HNO_2$$
$$2HNO_2 \rightarrow 2N_2O_3 + H_2O$$
$$N_2O_3 \rightarrow NO\bullet + NO_2\bullet$$

Ascorbate

$$NO_2^- + H^+ \rightarrow HNO_2$$
$$2HNO_2 + Asc \rightarrow 2NO\bullet + dehydroAsc + 2H_2O$$

Polyphenols (Ph-OH)

$$NO_2^- + H^+ \rightarrow HNO_2$$
$$Ph\text{-}OH + HNO_2 \rightarrow Ph\text{-}O\bullet + NO\bullet + H_2O$$

Mitochondral respiratory chain enzymes

$$NO_2^- + 2H^+ + e^- \rightarrow NO\bullet + H_2O$$

NO oxidation

$$2NO + O_2 \rightarrow 2NO_2$$
$$2NO + 2NO_2 \rightarrow 2N_2O_3$$
$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

$$NO + HbFe^{2+} \rightarrow NO_3^- + HbFe^{3+}$$

1.2 THE CHEMICAL BIOLOGY AND PHYSIOLOGY OF NO

Chemically, NO is a free radical consisting of one atom of nitrogen and one atom of oxygen, elements which are neighbours in the periodic table. The properties of NO being uncharged, lipophilic and very small, enables it to diffuse readily across cell membranes to reach specific target cells. NO has a high affinity to heme and other iron-containing moieties, exceeding that of oxygen. This property is essential for many of its biological effects and its regulation, including the activation of heme-containing guanylyl cyclase (sGC), and the rapid inactivation of NO by hemoglobin. The *in vivo* life time of the tiny free radical NO is very short as it undergoes rapid oxidation or reacts with other radicals. Thus it acts primarily in local environments in a paracrine or autocrine manner. The biological chemistry of NO and related reactive nitrogen oxide species is highly complex and involves many potential reactions including direct interaction and binding to targets such as metal complexes, or reactions with radicals to form other potentially bioactive nitrogen oxide species (RNS)[12]. A classic example is the ultrarapid reaction of NO with the superoxide anion radical (O_2^-) to form peroxynitrite ($ONOO^-$) (Box 1). Formation of peroxynitrite and its subsequent breakdown into highly reactive radical species (OH•, NO_2•) have been suggested to be responsible for many of the pathophysiological events associated with prolonged high NO generation in tissues[12,13].

NO is an essential signaling molecule in many aspects of mammalian biology. It plays a key role in vascular homeostasis and acts in various ways to regulate vascular tone, neurotransmission, platelet aggregation, redox signaling, cellular respiration and host defense. The paracrine actions of NO are typically mediated through activation of the sGC by binding to its heme group, with resulting activation and increased synthesis of 3, 5-cylclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in target cells[14-16]. This secondary messenger phosphorylates protein kinase G (PKG), which has multiple intracellular functions including the phosphorylation of ion channels and the inhibition of voltage-gated calcium channels resulting in a decrease in intracellular calcium, thereby promoting relaxation of vascular smooth muscle cells with subsequent vasodilation[17-20].

Classically, endogenous NO is synthesized from L-arginine and molecular oxygen by NOS synthases (NOSs) to generate L-citrulline and NO. NO synthases are found in essentially all cell types including vascular endothelial cells, macrophages, platelets, nerve cells, epithelial cells and more. The mammalian NO synthase family consists of a group of 3 isoforms including neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2). These enzymes are named after the cell type where they were first discovered. The catalytic process involves a 5-electron oxidation of L-arginine and enzyme activity requires the presence of cofactors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH4) and calcium-calmodulin, as well as the co-substrates nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. The enzymes are activated by a calcium-calmodulin complex. The bioactivity of nNOS and eNOS (constitutive isoforms) is strictly regulated by a number of transcriptional (expression and abundance) and post-translational (activity and function) mechanisms to maintain NO homeostasis, whereas iNOS (the induced isoform) is mostly modulated

transcriptionally. Thus, once iNOS is expressed, it generates large quantities of NO over a prolonged period of time.

As mentioned above the reactive nature of NO renders it to oxidize quickly and this represents an additional very important control mechanism of NO bioactivity. The two major end products of NO metabolism are the oxidation products nitrite and nitrate. Ultimately the vast majority of endogenous NO is oxidized to nitrate. Because NO itself is difficult to measure directly, nitrate and nitrite have been used as surrogate markers of NO formation. Among researchers these inorganic anions have generally been considered completely inert. However, this view is now being seriously challenged by recent discoveries convincingly showing that nitrite and nitrate anions can in fact be reduced back to bioactive NO in blood and tissues[6,21-24]. Characterization of this nitrate-nitrite-NO pathway is a central part of the present thesis.

1.3 GENERATION OF NO BY BACTERIA

Compared to eukaryotic cells, the prokaryotic cells are vastly superior in adapting their biochemical capabilities in response to changes in the environment. Mammals are strictly dependent on oxygen as the terminal electron acceptor for respiration. Bacteria on the other hand can use a variety of other compounds for respiration and two classical examples are nitrate and nitrite. These molecules can function as alternative electron acceptors, allowing bacteria to respire and survive under anoxia. Thus, bacteria can thrive in extreme places ranging from hot sulphur-containing springs, in the deepest oceans, to the top of mountains. Mammals are also inhabited by bacteria. In fact, it has been estimated that >90% of all cells in an adult human are bacteria, with the highest densities in the oral cavity and the large intestine[5]. Bacteria have a predominant role in the biological nitrogen cycle since they catalyze most reactions. In this cycle (Figure 1 A), inorganic nitrogen is converted to a biologically useful form in processes known as nitrification - the oxidative conversion of ammonia to nitrate, and denitrification - a respiratory process whereby nitrate is successively reduced anaerobically to nitrite, nitrous oxide and finally dinitrogen gas. Life would be impossible if the combined processes of nitrification and denitrification were not compensated by a third process, dinitrogen fixation, which completes the nitrogen cycle with release of nitrogen gas back to the atmosphere[25].

In the denitrification part of the nitrogen cycle in nature, NO is an obligate intermediate and it is produced by bacterial nitrite reductases[5,26]. One part of this thesis examines the possibility that NO can be formed also from bacteria that thrive in the gastrointestinal tract. The occurrence of such NO formation and its possible biological significance will be discussed later.

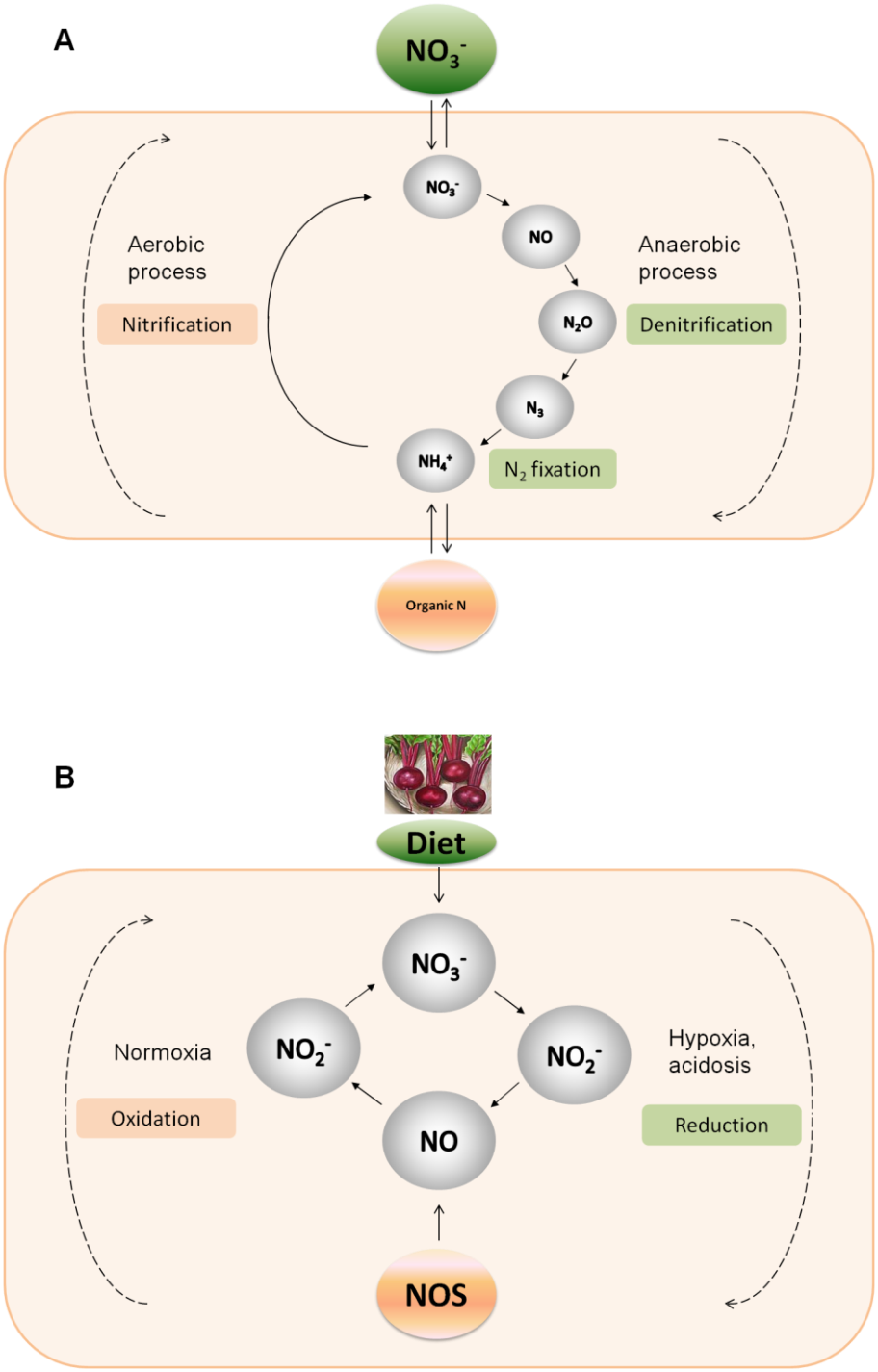


Figure 1. Schemes illustrating the classical nitrogen cycle in nature along with a recently described mammalian nitrogen cycle. (A) In the nitrogen cycle in nature atmospheric nitrogen gas is fixed and the ammonia formed is oxidized to nitrate in an aerobic process known as nitrification. In another process termed denitrification, anaerobic bacteria catalyze the serial reduction of nitrate to nitrite, NO, nitrous oxide and finally dinitrogen gas, which is released back to the atmosphere thereby completing the cycle. (B) In mammals a similar nitrogen cycle has been discovered. NO generated from the NOS pathway is rapidly oxidized to nitrite and then nitrate. In a situation of hypoxia and acidosis, nitrate is reduced back to nitrite and then NO.

1.4 DISCOVERING NO SYNTHASE-INDEPENDENT NO FORMATION IN MAMMALS

Until recently it was generally thought that the only source of endogenous NO generation in mammals is the L-arginine-NOS pathway. The classic NO synthase-dependent pathway requires a complex 5 electron oxidation of the guanidine nitrogen of L-arginine and a number of co-factors to form NO. However, in 1994, two independent research groups showed that NO and other reactive nitrogen oxides were formed non-enzymatically in the stomach following protonation of nitrite in the acidic stomach[6,21]. The NOS-independent pathway turned out to be fundamentally different. The reaction uses the simple inorganic anion nitrite (NO_2^-) as a substrate instead of L-arginine, and the reaction involves only a one-electron reduction to NO (Figure 1 B). Nitrite reduction to NO in the stomach is strictly acid dependent and can be abolished by proton pump inhibitors that increase gastric pH[21]. The levels of NO in the human stomach are very high (10-100 ppm), i.e. several orders of magnitude higher than those required for vasodilation. Does this generation of NO in the gastric lumen have any biological significance? This question is yet to be fully resolved but studies have shown that the high levels of gastric NO may play a role in maintaining gastric integrity. First, it may be a part of first-line host-defense utilizing the potent antimicrobial effects of NO and related nitrogen oxides. Interestingly, *E. coli*, *Salmonella*, *Shigella* and other enteropathogens are remarkably resistant when exposed to acid alone *in vitro*[27]. However, those same pathogens can be killed when exposed to a mixture of nitrite and acid, thereby simulating the natural mixing of nitrite-containing saliva and acidic gastric juice[6]. Later studies show that the combination of authentic human gastric juice and nitrite-rich saliva inhibits a variety of pathogens[28]. The antibacterial effects of nitrite in the gastric lumen are likely due to multiple reactive nitrogen intermediates (RNIs) generated from this anion under acidic conditions. In addition to pH and nitrite concentrations, many other factors determine the magnitude of NO and RNI generation in the GI tract, including the presence of reducing agents e.g. vitamin C, thiocyanate, polyphenols, proximity to heme groups, proteins, thiols, as well as the oxygen tension[29].

Nitrite-derived gastric NO may also affect the host mucosa. Indeed, studies have shown that nitrite-rich saliva increases mucosal blood flow and mucus generation in an NO dependent manner[30,31]. These effects are associated with strong gastroprotective effects. As an example, in rats the ulcerogenic properties of orally administered Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are strongly attenuated by dietary nitrate, which greatly increased intragastric NO formation after conversion to nitrite in saliva[32].

1.5 WHERE IS THE NITRITE AND NITRATE COMING FROM?

The inorganic nitrite and nitrate in our bodies stem from two major sources: the endogenous L-arginine-NOS pathway and the diet[33]. As discussed above endogenously generated NO rapidly oxidizes to form nitrite and nitrate in blood and tissues. The reaction of NO in blood with oxyhaemoglobin produces nitrate and methaemoglobin,

while nitrite is the major oxidation products in cells. Our diet represents the second major source of nitrate and nitrite. Green leafy vegetables such as spinach, beetroot and lettuce naturally contain large amounts of nitrate, whereas cured meats and bacon are particularly rich in nitrite which is added for preservation purposes, to enhance the appearance and to prevent from botulism[34]. Traditional Mediterranean, Chinese or Japanese diets are very rich in vegetables and will provide much more nitrite and nitrate compared to the Western-type diet. Drinking water may also contain variable amounts of nitrate although in most countries the levels are strictly regulated.

Normal plasma nitrate levels are in the range of 10-40 µM, whereas nitrite levels are typically 100-1000 fold lower (50-300 nM). The actual nitrite and nitrate levels in blood or plasma show significant variability depending on differences in dietary habits, life style, disease states and physical exercise. Recent reports show that plasma nitrite levels of Tibetan, high altitude inhabitants well adapted to environmental hypoxia, are approximately 10 µM, i.e. 100-fold higher than in people living at sea level[35]. The suggested reason for this is an upregulation of vascular eNOS in response to the hypoxic environment. In systemic inflammatory situations such as sepsis or in gut inflammation such as severe gastroenteritis[36] or celiac disease[37], nitrite and nitrate levels can be greatly elevated owing to massive iNOS induction. In contrast, in a situation of endothelial dysfunction and reduced eNOS activity, plasma nitrite and nitrate levels are lower, as in patients with atherosclerosis and in eNOS knock-out mice[38].

1.6 THE ENTRO-SALIVARY CIRCULATION OF NITRATE

After intake of nitrate-rich food such as spinach or beet roots, nitrate is rapidly absorbed in the gastrointestinal tract and then enters the circulation where it mixes with endogenous nitrate from the NOS pathway. The level of plasma nitrate reaches a peak within 60 min after nitrate ingestion and the half-life in plasma is 5-6 h. About 25 % of circulating nitrate is actively extracted by the salivary glands and concentrated in saliva[39,40]. The reason for this exceptional 10-20 fold accumulation of nitrate in saliva is still unclear. Commensal facultative anaerobic bacteria in the mouth reduce parts of the nitrate to nitrite by the action of nitrate reductases[5,41]. The formed nitrite is continuously swallowed and can enter the systemic circulation after absorption[39].

1.7 SYSTEMIC NO GENERATION FROM NITRITE

In 1995, a year after the original discovery of NOS-independent NO generation, Zweier and colleagues reported that N^{15}-labeled nitrite was reduced to NO in rat ischemic heart muscle and the NO generation in this model could not be effectively blocked by NOS inhibitors[22]. This indicated that nitrite-reduction may be a ubiquitous phenomenon applicable also to the tissues. While the first studies in the field focused on non-enzymatic nitrite-reduction, subsequent studies revealed that there are also enzymes capable of the one-electron reduction of nitrite to NO[42]. The list of mammalian nitrite reductases is rapidly increasing and it includes xanthine oxidoreductase[43-46], enzymes of the mitochondrial chain[47], deoxygenated hemoglobin/myoglobin (deoxy-

Hb/Mb)[23,48-50], aldehyde oxidase, cytochrome P450s[51] and even the NOS itself[52]. Interestingly, while the NOSs are oxygen-dependent and therefore dysfunctional in hypoxic situations, the nitrite reduction pathways are instead greatly enhanced under these conditions.

1.8 XANTHINE OXIDOREDUCTASE – NOVEL NITRITE REDUCING PROPERTIES

Xanthine oxidoreductase (XOR) is a molybdoflavin enzyme that is widely distributed in mammalian tissues and it catalyzes the terminal two steps of purine degradation from hypoxanthine to xanthine and xanthine to uric acid. XOR is also well known as an important source of superoxide and reactive oxygen species (ROS) generation, not only in pathological conditions such as tissue ischemia, vascular inflammation or infection, but also in signal transduction[53-55]. There are two forms of XOR: xanthine dehydrogenase (XDH), which is the dominant form in tissues and xanthine oxide (XO). XDH is a single gene product transcribed from XOR, whereas during pathological conditions, XDH can be converted to XO by post-translational modification involving oxidation of cysteine residues or limited proteolysis[56,57]. The differences of structure conformation and electrostatic microenvironment surrounding the FAD cofactor result in XO with higher affinity to O_2 compared to XDH[58]. Both XDH and XO catalyze the reactions hypoxanthine to xanthine and xanthine to uric acid. However, XDH requires NAD^+ as an electron acceptor for the reductive process and generates a stable product NADH, whereas XO is not able to use NAD^+ as an electron acceptor. Instead, for this purine oxidation process XO reduces molecular oxygen and thereby generates the highly reactive molecules superoxide and hydrogen peroxide[53,59,60]. The enzyme activity of XOR requires molybdopterin, iron-sulphur centers and FAD as cofactors to transfer electrons from xanthine to oxygen and NAD^+, yielding superoxide, hydrogen peroxide and NADH. Of interest to the current thesis is the more recent finding that XOR can donate electrons not only to oxygen (to form superoxide) but also to nitrite with resulting reduction and possible formation of NO. Interestingly, XOR is a molybdenum-containing enzyme with structural similarities to the bacterial nitrate reductases[46,61]. A specific aim of this thesis has been to explore if also the much more stable nitrate anion can be reduced by XOR, thereby forming nitrite. Such a pathway could then theoretically contribute to nitrite and NO formation in mammals in addition to the NO synthases.

1.9 CARDIOVASCULAR EFFECTS OF NITRITE

Nitrite has been known for its vasodilatory properties for more than half a century, i.e. long before the NO pathway was even discovered. However, the nitrite concentrations and acidity used in these early pharmacological studies were far outside physiological levels seen in tissues[62]. In 2001, Modin et al. showed that near-physiological concentration of nitrite can generate NO and dilate rat aortic rings in a buffer of pH 6.6, which is an acidity commonly seen in tissues during ischemia[24]. At the same time Gladwin et al. showed that nitrite levels in the human forearm circulation dropped from artery to vein, indicating the consumption of nitrite across the circulation[63]. The consumption of nitrite was increased during forearm exercise, a situation when blood

oxygen tension is reduced. These studies suggested an active role of nitrite in vasoregulation. Gladwins group later went on to demonstrate vasodilation in humans after infusion of near-physiological amounts of nitrite in the forearm[23]. The same group hypothesized that nitrite reduction occurred in the blood and that it was catalyzed by deoxygenated hemoglobin in an oxygen tension-dependent fashion. The mechanism for nitrite reduction in blood and tissues and its possible role as a physiological regulator of hypoxic/ischemic vasodilation has been the matter of extensive research ever since and has been discussed in detail in several recent reviews[7,64,65] as well as in this thesis.

Although the occurrence of endogenous NO generation from nitrate and nitrite has been clearly established and its physiological importance is starting to be explored, there are several questions remaining. Many of these relate to basic chemical biology, including mechanisms of NO generation in different tissues, controls of production and sites of release. In this thesis some of these areas have been investigated, in particular we have attempted to better clarify host-bacterial interactions in control of nitrate-nitrite-NO homeostasis.

2 AIMS OF THE PRESENT THESIS

The overall aim of the present thesis was to further characterize NO generation from the recently discovered nitrate-nitrite-NO pathway and its physiological and therapeutic potential.

More specifically the aims were:

1. To study if commensal bacteria in the gut can generate NO *in vivo*.
2. To explore if mammalian tissues are capable of reducing nitrate and its biological consequences.
3. To study the role of xanthine oxidase in nitrate and nitrite homeostasis.
4. To study if a cross-talk exists between NOS-independent and NOS-dependent pathways in control of endogenous NO homeostasis.

3 MATERIAL AND METHODS

Below follows a brief description of the methods used in this thesis work. For a more extensive account, the reader is referred to the individual papers.

3.1 ANIMAL EXPERIMENTS

3.1.1 NO measurements in the gastrointestinal tract (Paper I)

Adult male rats were divided into four groups; supplemented with live *lactobacillus rhamnosus* (LGG) alone; LGG together with sodium nitrate (0.1 mmol kg^{-1} day^{-1}) (LGG + Nitrate); sodium nitrate alone (0.1 mmol kg^{-1} day^{-1}) (Nitrate) or sodium chloride alone (0.1 mmol kg^{-1} day^{-1}) (Control) for 7 days. At the day of measurements, anesthesia was performed by administration of sodium pentobarbital (60 mg kg^{-1}) intra-peritoneally (i.p.), followed by laparotomy. Different volumes of NO-free air were inflated into the caecum (5 ml), stomach (4 ml), small intestine (2.5 ml), and colon (3 ml) of rats using a 5 ml syringe with a thin needle. NO-free air was obtained by sampling room air via a charcoal filter. External clamps were used to prevent the air from passing into neighboring compartments. The air from different compartments was incubated for 15 sec, then aspirated and immediately injected into a chemiluminescence analyser (Aerocrine AB, Stockholm, Sweden) and the peak NO concentration was measured.

3.1.2 Nitrate administration *in vivo* (Paper II, III, IV)

In the animal experiments of paper II, sodium nitrate (10 mg kg^{-1}) or placebo (NaCl) were given i.p. to germ-free mice, C57BL/6 wild-type ($NOS3^{+/+}$) and eNOS-deficient mice ($NOS3^{-/-}$). In paper III, the same concentration of sodium nitrate or sodium chloride was given i.p. to germ-free and conventional (NMRI) mice. One group of germ-free animals was pre-supplemented with the XOR inhibitor allopurinol (100 mg kg^{-1} day^{-1}) via the drinking water, before nitrate administration. After 1-2 hours, the animals were killed and blood and tissue samples were collected.

In the long-term animal experiments of paper IV, the treatment group was given two doses of sodium nitrate in their chow (0.14 g or 1.4 g $NaNO_3$ kg^{-1}) to achieve a daily intake of 0.1 mmol and 1.0 mmol nitrate kg^{-1} day^{-1}, respectively. The placebo group was supplemented with a standard chow. The supplementation of nitrate was carried out for 9 weeks. At the end of the experiment, animals were anaesthetized and a catheter was placed in the left carotid artery for blood sampling. The blood and tissue samples were collected.

3.1.3 Blood and tissue sample collection (Paper II, III, IV)

Blood samples were collected into tubes containing *N*-ethylmaleimide (NEM) (final concentration in 5 mM) and EDTA (final concentration in 2 mM). The blood was centrifuged immediately at 370-400 g for 5-10 min at 4^{o} C and stored at -80^{o} C until analysis. After blood sampling, the animals were sacrificed and liver, kidney, aorta, heart, lung, stomach, small intestine, colon and skeleton muscle samples were immediately removed and snap frozen on dry ice and stored at -80^{o} C for later analysis.

3.1.4 Blood pressure measurements (Paper II, IV)

In the rat experiments of paper II, anesthetized animals received an intravenous bolus dose (10 mg kg^{-1}) of sodium nitrate or placebo and continued infusion of sodium nitrate or placebo for 60 min followed by an intravenous bolus dose (50 mg kg^{-1}) of the NOS inhibitor L-NAME (Sigma-Aldrich). Then the blood pressure was monitored during 10 min with a Grass Polygraph (Grass Instrument Co., Quincy, Mass., U.S.A.) and arterial blood gases were measured using a clinical blood gas analyzer (ABI 505, Radiometer).

In the rat experiments of paper IV, telemetric measurements were used to measure blood pressure. Animals were anaesthetized with inhalation of isoflurane (2.2 %) which continued throughout the surgery. A telemetric device (PA-C40) (DSITM, Transoma Medical, St Paul, MN, USA) was implanted into the aortic lumen as described previously[66]. Animals were allowed to recover for 10 days after surgery. The measurement of blood pressure and heart rate was conducted throughout a control period (72 h), then during L-NAME treatment (72 h) and finally after the abrupt termination of the long-term nitrate administration.

3.1.5 Ischemia/reperfusion (Paper II)

In the rat experiments of paper II, anesthetized animals received an intravenous bolus dose of sodium nitrate (10 mg kg^{-1}) or placebo (NaCl) diluted in PBS (pH 7.4) followed by intravenous infusion of the same (3 mg kg^{-1} h^{-1}) at an infusion rate of 3 ml h^{-1}. 60 min after the addition of sodium nitrate or placebo, L-NAME (50 mg kg^{-1}) was given to animals as an intravenous bolus dose. 10 min after L-NAME administration, a suprarenal clamping of the abdominal aorta was performed, followed by a 30 min period of ischemia. The clamp was then released and the abdominal aortic blood flow was monitored using a Transonic flow probe 2SB, T206 (Transonic System Inc.).

3.1.6 Studies in newborn infants and *in vitro* experiments with bacteria (Paper I)

A total number of 34 healthy, newborn infants (14 girls/20 boys) were included in the study. Colonic gas samples were collected using a minimally-invasive tonometric balloon technique by inserting an all-silicone catheter equipped with an inflatable balloon tip 8-10 cm into the sigmoid colon via the rectum[67]. The balloon was inflated with 5 ml NO-free air and left to equilibrate in the intestine for 5 min, the air was then aspirated and immediately injected into the chemiluminescence NO analyzer to measure NO levels.

Lactobacilli sp, E. coli, *Bifidobacterium sp* and *Staphylococcus aureus* isolated from faeces of two healthy neonates were incubated anaerobically at 37^{o} C for 24-48 hours on different agar plates supplemented with 0.1 mM sodium nitrate. 100 µl pre-cultured inoculates was put on either *lactobactcilli* agar AOAC for *lactobobacilli* and *bifidobacteria*, ISO-sensitest agar plates for *E. coli* and *S. aureus*. After inoculation, the plates were placed in infusion bags together with an anaerobic pouch system and an anaerobic indicator. The bags were sealed and injected with 300 ml air for 1 hour to achieve the anaerobic condition. The anaerobic pouch inside the bags was then sealed off and isolated from the plates since preliminary experiments showed that considerable

NO consumption by the anaerobic pouch system. The gas-tight bags were incubated at 37^{o} C and after 1, 6, 9, 18, 24 hours, gas (10 ml) was aspirated and NO concentration was immediately measured by chemiluminescence.

3.1.7 Studies involving human volunteers (Paper II)

To study systemic nitrate reduction in humans, 4 healthy volunteers ingested sodium nitrate (10 mg kg^{-1}) orally. To avoid any contribution from bacterial nitrate reduction by oral commensal bacteria, the subjects rinsed their mouths with an antimicrobial mouthwash solution (Corsodyl, Glaxo-SmithKline) immediately before the nitrate load. Plasma and salivary samples were taken repeatedly and nitrate and nitrite levels were measured.

3.1.8 Levels of NO, nitrite and nitrate (Paper I, II, III and IV)

3.1.8.1 Chemiluminescence assay for NO (Paper I, II)

The chemical basis for this chemiluminescence assay is the reaction between NO and ozone (O_3), which yields nitrogen dioxide that is partially in the excited stage (NO_2*). When NO_2* returns to its ground-state, the light is emitted in the near-infrared region of the spectrum and can be detected by a photosensitive surface and subsequently amplified by a photomultiplier tube. The intensity of this luminescence is then converted into an electric signal. To measure levels of NO, the gas sample is removed at a constant flow rate and immediately mixed with an excess of O_3 in the evacuated reaction chamber. The subsequent fast reaction with O_3 allows detection of rapid fluctuations in NO concentration. This chemiluminescence assay of NO is highly sensitive, with a detection limit of 1 parts per billion (ppb), and the amount of light emitted is directly proportional to concentrations of NO between 1 and 100,000 ppb. Interference by other gases, including other nitrogen oxides, is minimal.

The concentrations of nitrite and nitrate were determined by a chemiluminescence assay after reductive cleavage and subsequent release of NO into the gas phase (Paper I, II, III). To measure nitrite and nitrate concentrations, samples were introduced via a gastight syringe into a purged reaction vessel containing the reducing solution and coupled to a condenser. The temperature of the reaction vessel was controlled by a heating jacket unit (Sievers, Boulder, Co., USA) through which warm water from a constant-temperature bath circulated. A constant flow of nitrogen served as the carrier gas for NO. As a final step, the gas was bubbled through sodium hydroxide (1M 0^{o}C) to trap any reminding traces of acid prior to introduction into the NO analyzer (Aerocrine AB, Stockholm, Sweden). The flow rate from the reaction vessel was adjusted with a needle valve. The data obtained were further analyzed with the Windows Azur platform and the levels of nitrite and nitrate were calculated and reported in M (mol/L) by comparing the areas under the curve with known concentrations of nitrite or nitrate.

3.1.8.2 High performance liquid chromatography (HPLC) determination (Paper IV)

In paper IV, nitrite and nitrate levels were measured by another sensitive and selective measurement, HPLC system (ENO-20, Eicom, Japan), which uses reverse phase chromatography to separate nitrite from nitrate and then nitrate is reduced to nitrite

through a reaction with cadmium and reduced copper inside a reduction column. Reduced nitrite is then derivatized with a Griess reagent and the level of diazo compounds is measured by a visible detector at 540 nm.

3.1.8.3 Diaminofluorescein-2 (DAF-2) assay for NO (Paper II)

To measure NO production over time in liver tissue homogenates incubated with sodium nitrate and nitrite, a DAF-2 assay was used *in vitro*. The principle of this assay is that DAF-2 is oxidized by the NO reaction product NO_2 whereby the diamino complex is oxidized to an aromatic radical yielding the highly fluorescent product DAF-2 triazole. The change in fluorescence was measured at excitation 485nm and emission at 538nm during 15 h at 37^{o} C in a micro plate reader (Molecular Devices).

3.1.9 Tissue nitrate reductase activity (Paper II, III)

Tissues were homogenized in 3 parts in 10 mM Tris-HCl pH 7.4 containing 250 mM sucrose using a polytron (Kinematrica) on ice or the Bullet Blender^{TM} (Next Advance, Inc., NY, USA). Supernatant protein concentrations of homogenates were determined by a Bradford assay (BioRad). Protein (7 mg ml^{-1}) was incubated with a mixture of cofactors including NADPH, UDP glucuronic acid (UDPGA), glutathione (GSH), NAD^+ and NADH in phosphate buffer (pH 7.4) with or without 300 µM $NaNO_3$ and 2 mM allopurinol. 100 µl of this mixture was taken and nitrite was measured immediately to represent time 0. The rest of the mixture was deoxygenated with a stream of N_2 (0 % oxygen) for 2 min, sealed and incubated for 60 min at 37 ^{o}C and the levels of nitrite were measured subsequently. A tissue pool from 3-5 animals was used for each experiment and data represent the mean of at least three experiments.

3.1.10 Western blot analysis (Paper III, IV)

For quantification of XOR (Paper III) and phosphorylated-eNOS (Paper IV) protein expressions, frozen tissue samples from animals were homogenized using a power homogenizer (KEBO-lab, Stockholm, Sweden, Paper III) or a Bullet Blender^{TM} method (Next Advance, Inc., NY, USA, Study IV) utilizing 0.5 mm stainless steel silicate beads (Next Advance, Inc., NY, USA) in 500 µl lysis buffer as described earlier[68]. Protein concentrations were determined by means of a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of total protein were separated on 7.5 % SDS-PAGE gels and transferred to either nitrocellulose or polyvinylidene difluoride (PVDF) membranes. Blots were probed with a rabbit polyclonal antibody against XOR (Rockland, Gilbertsville, PA, USA, Paper III) or rabbit polyclonal antibody against phosphorylated eNOS (Santa Cruz Biotechnology, CA, USA, Paper IV) and a mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology, CA, USA) as an endogenous control. Labeling was detected by SuperSignal West Pico chemiluminescence substrate (Themo Scientific, Rockford, IL, USA). Images were analyzed by a luminescent image analysis system LAS 1000+ (Fujifilm, Kanagwa, Japan). The results were quantified by densitometry and reported as relative optical density of the specific proteins.

3.1.11 Amino acid measurements (Paper IV)

To analyze arginine, citrulline and ornithine levels in plasma, an HPLC method was used as described in detail previously[69] with modified chromatographic separation conditions[70]. Samples were cleaned up by solid-phase extraction on polymetric cation-exchange columns using monomethylarginine as an internal standard and derivatized with orthophthadildhyde reagent containing 3-mercaptopronicacid. Chromatography was performed by isocratic reversed-phase HPLC with fluorescence detection. For all data analysis the intra- and inter-assay coefficients of variation (CV) were < 1.5% and < 3.5%, respectively.

4 STATISTICAL ANALYSIS

Data processing was performed with GraphPad Prism software 4.0 or 5.0 (GraphRad Software, Inc. La Jolla, USA). All data are shown as means ± SEM. Single comparisons between parameters were tested for significance with Mann-Whitney test or two-tailed independent Student's *t* test. Correlation was analyzed with the Spearman rank test. For multiple comparisons, ANOVA with the Bonferroni post hoc test or Dunnett's multiple comparison test was used. $*P < 0.05$ was considered significant.

5 RESULTS

5.1 NO GENERATION BY COMMENSAL BACTERIA IN THE GASTROINTESTINAL TRACT (PAPER I)

We measured NO levels in the small intestine, caecum and colon of rats supplemented with LGG in combination with sodium nitrate or saline as control. The NO levels were increased 3-8 times in the small intestine and caecum in animals supplemented with LGG and sodium nitrate compared to the control animals. The levels of NO in the caecum were increased in rats with LGG alone, whereas the NO in the small intestine did not change in those supplemented with nitrate alone (Figure 2).

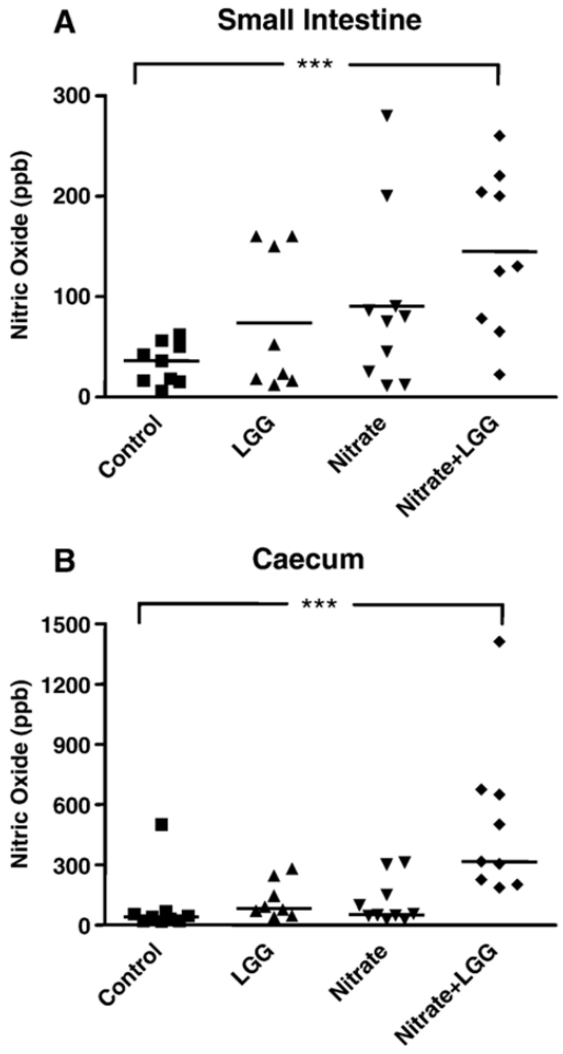


Figure 2. NO formation in the GI tract. Levels of NO in the small intestine (A) and caecum (B) of rats supplemented with *Lactobacillus rhamnosus* (LGG), sodium nitrate (0.1 mmol kg^{-1} day^{-1}), *Lactobacillus rhamnosus* + nitrate (LGG + nitrate) or control (saline). Data represent means ± SEM. ***$P < 0.001$ compared to controls.

The nitrite levels in the small intestine and caecum were significantly higher in rats supplemented with sodium nitrate, alone or in combination with LGG. In addition, NO levels in the caecum correlated to nitrite levels in the caecum (Figure 3).

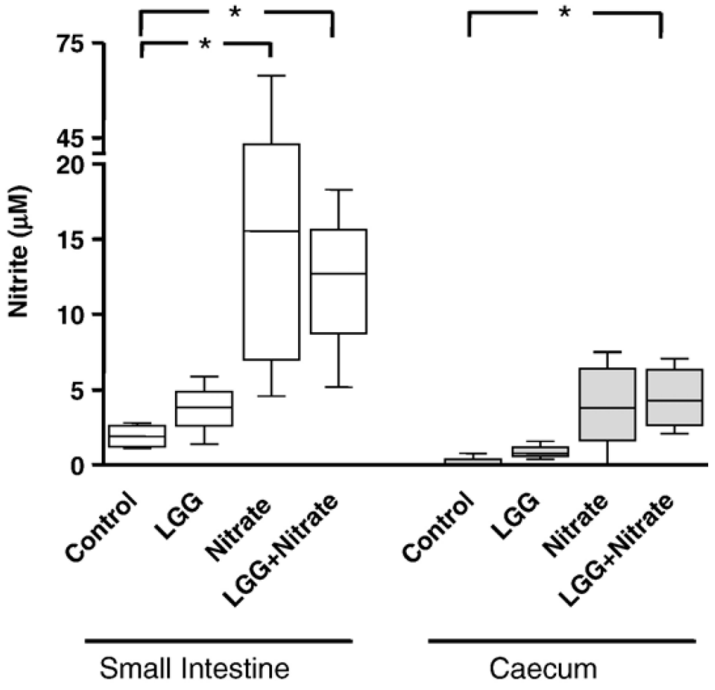


Figure 3. Nitrite levels increase in the GI tract after supplementation with nitrate and probiotic bacteria. Levels of nitrite in the small intestine and caecum of rats supplemented with *Lactobacillus rhamnosus* (LGG), sodium nitrate (0.1 mmol kg^{-1} day^{-1}), *Lactobacillus rhamnosus* + nitrate (LGG + nitrate) and control (saline). Data represent means ± SEM. **P* < 0.05 compared to controls.

When measuring NO generation in newborn infants (Paper I), we found that NO levels in colon correlated to nitrite concentrations in breast milk and faeces, but not to nitrate levels.

To study NO generation and the interaction among commensal bacteria (Paper I), we grew *lactobacilli*, *bifidobateria*, *E. coli* and *S. aureus* alone or together on nitrite-supplemented agar plates and measured NO formation. The *lactobacilli* and *bifidobacteria*, generated NO but *E. coli* and *S. aureus* did not. Moreover, when *lactobacilli*, *bifidobacteria* were co-incubated with *E. coli* or *S. aureus*, NO generation was decreased compared to those seen when *lactobacilli* and *bifidobateria* were grown alone indicating NO consumption by *E. coli* and *S. aureus*. Indeed, when exogenous NO gas (NO < 10 ppb) was injected to the bags of *S. aureus*, the NO levels were dramatically reduced in less than 8 h (Figure 4).

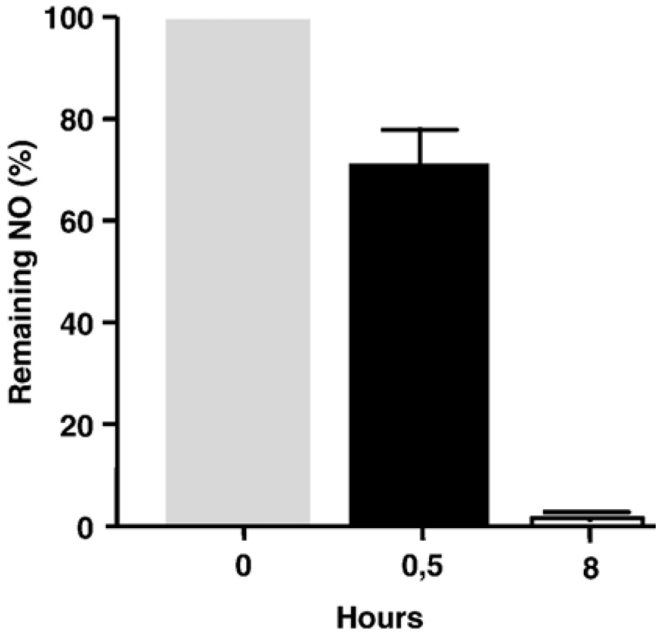


Figure 4. NO generated in the gut is consumed by other microbes. Consumption of NO by *S. aureus* grown anaerobically on agar plates at different time points.

5.2 A MAMMALIAN NITRATE REDUCTASE (PAPER II, III, IV)

5.2.1 Mammalian tissue enzymes generate nitrite from nitrate *in vitro* (Paper II, III)

It is known that bacterial nitrate reduction is extremely effective but the general view has been that mammalian cells cannot metabolize this anion. This thesis demonstrates the existence of a mammalian nitrate reductase that is active under normoxic conditions *in vitro* as well as *in vivo*.

To study mammalian tissue nitrate reduction (Paper II), we incubated mouse liver homogenates with sodium nitrate under anaerobic conditions at 37^{o} C for 60 min and then measured nitrite levels. Nitrate reduction was clearly demonstrated by the accumulation of nitrite and this activity was heat sensitive, indicating an enzymatic reaction (Figure 5 a, b, c, d). Nitrate reduction was present in liver homogenates from rats, mice and humans, with higher activity in rodents (Figure 6 a). Surprisingly, nitrate reduction was present not only during hypoxic conditions but also at physiological oxygen concentrations (Figure 5 b).

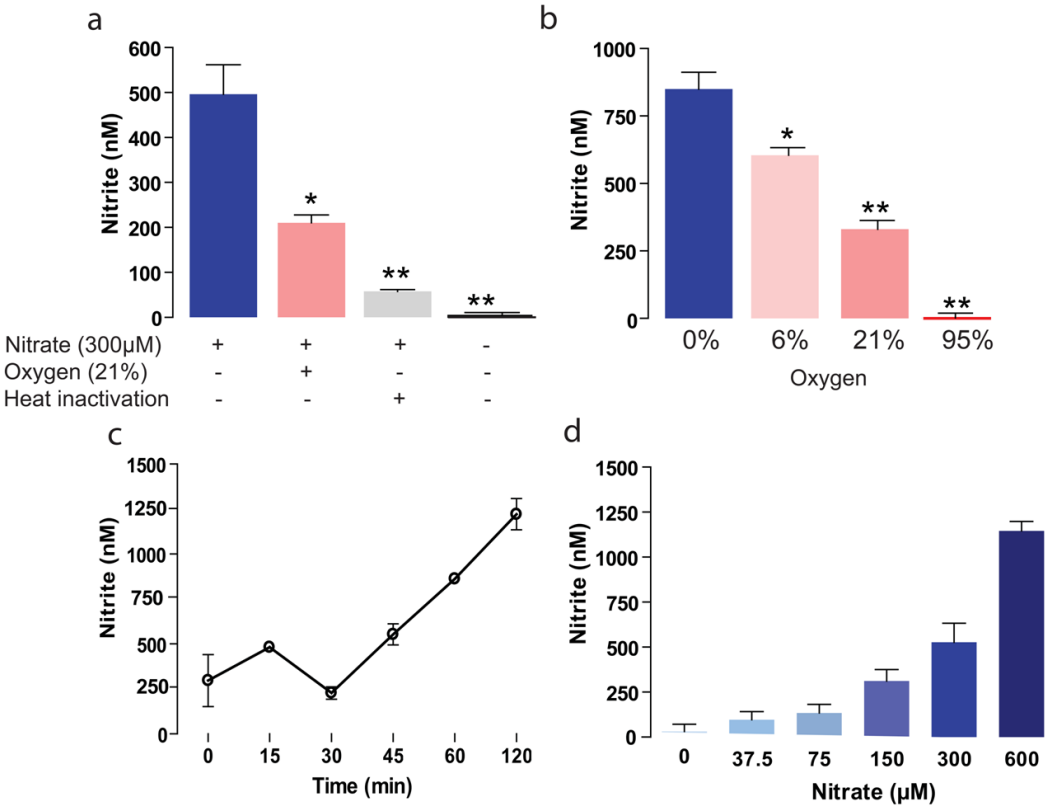


Figure 5. Mammalian nitrate reductase activity *in vitro* in mouse liver homogenates. (a) Nitrite formation after 60 min incubation in mouse liver homogenates with sodium nitrate (300uM) under anaerobic conditions, in the presence of oxygen, after heating the homogenate or without addition of sodium nitrate. (b) Nitrate reduction in liver homogenates with sodium nitrate and varying concentrations of oxygen. The nitrite levels were measured after 60 min incubation. (c) Time-dependent nitrite generation in the presence of sodium nitrate under anaerobic conditions. (d) Dose-dependence nitrite generation in the presence of sodium nitrate under aerobic conditions. Data represent means ± SEM. *P < 0.05 and **P < 0.01 compared to controls or 0% oxygen or zero point time.

We also performed experiments looking at nitrate reduction in different mouse organs and found that nitrate reductase activity was present in colon, small intestine, stomach, liver, kidney, heart and lung, with the highest activity in gastrointestinal tissue and lowest in heart and lung (Figure 6 b). Notably, in most tissue homogenates the nitrate reductase activity was attenuated in the presence of allopurinol, an inhibitor of XOR (Figure 6 a, b). In the presence of the NO synthase inhibitor L-NMMA, nitrite formation was unaffected, suggesting that NO synthases are not involved.

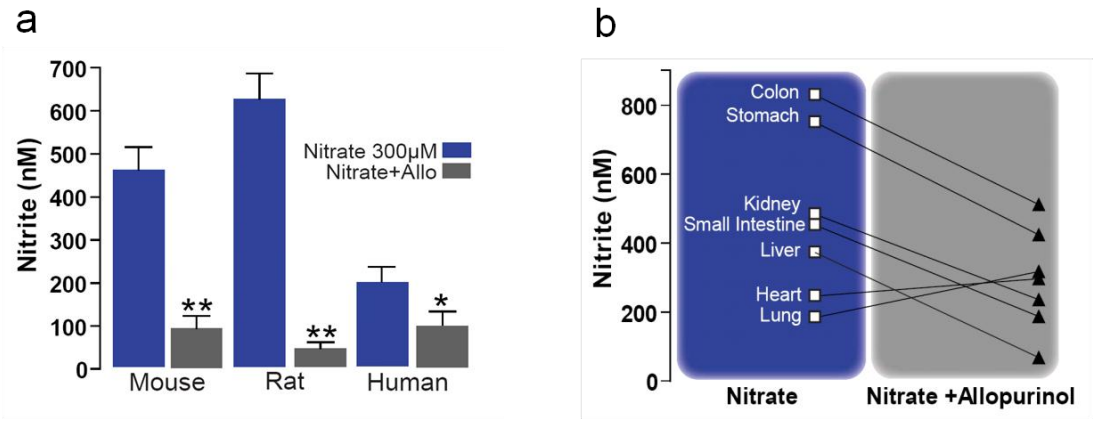


Figure 6. Mammalian nitrate reductase activity in different species and in a number of different organs. (a) Nitrate reductase activity in liver of rodents and humans measured as nitrite accumulation in liver homogenates after 60 min of anaerobic incubation with sodium nitrate (300 uM). The generation of nitrite was inhibited by the xanthine oxidoredctase inhibitor allopurinol (2 mM, Nitrate + Allo). (b) Nitrate reductase activity in different organs of mice. Tissue homogenates were anaerobic incubated with sodium nitrate and the changes in nitrite concentrations were measured after 60 min. The generation of nitrite was significantly inhibited by allopurinol (2 mM) in all measured organs except the lung and heart. *P < 0.05 compared to control. Data represent means ± SEM. *P < 0.05 and **P < 0.01 compared to nitrate alone.

To study nitrate reduction in germ-free animals (Paper III), we incubated mouse gastro-intestinal tissue homogenates with nitrate using the same protocol as we did for the liver and other tissues. Nitrite formation was even higher in germ-free mice compared to conventional mice (Figure 7), indicating an upregulated nitrate reductase activity in mice completely lacking bacteria.

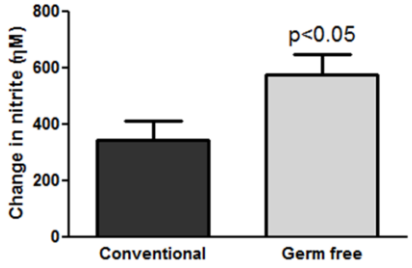


Figure 7. Nitrate reductase activity is enhanced in gastro-intestinal tissues of germ-free mice. Changes in nitrite were measured after 60 min incubation of the mouse gastro-intestinal homogenates with sodium nitrate under anaerobic conditions.

5.2.2 Nitrate reduction *in vivo* (Paper II, III, IV)

A bolus dose of sodium nitrate increased plasma nitrite levels during normoxic conditions in rats and this increase was attenuated by 40% in the presence of the XOR inhibitor allopurinol (Paper II) (Figure 8 a, b). This again suggests that XOR catalyzes

the reduction of nitrate *in vivo*, during normoxic conditions. However, the nitrate reductive activity was not completely inhibited by allopurinol suggesting the presence of other yet unidentified enzyme pathways for nitrate reduction in mammals.

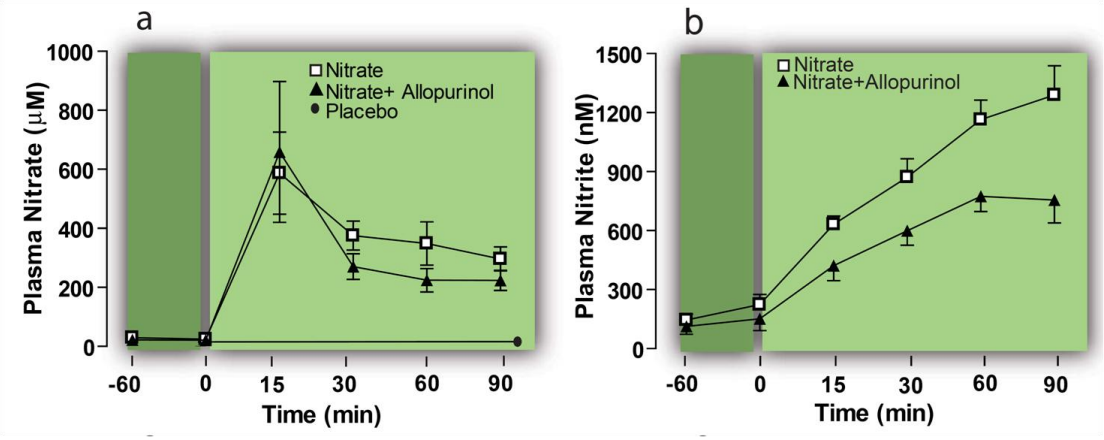


Figure 8. Nitrate reductase activity *in vivo* in mice. Plasma nitrate (a) and nitrite (b) concentrations increased after an intravenous infusion of nitrate. Anesthetized rats (n = 11) were given i.v. an bolus dose of sodium nitrate (10 mg kg^{-1} body) and blood samples were collected at the indicated time points. 7 additional rats were given allopurinol 30 mg kg^{-1} i.p. before sodium nitrate infusion and then blood samples were collected. Data represent means ± SEM. **P* < 0.05 compared to nitrate infusion before.

In humans, plasma nitrite level also increased after an oral nitrate load (Paper II). To exclude any contribution from oral nitrate reduction by oral commensal bacteria, subjects rinsed their mouth with an antibacterial mouthwash solution before nitrate intake. Despite abolishing salivary nitrite formation after the mouthwash, plasma nitrite levels increased 50-70 % after nitrate ingestion (Figure 9). This suggests the existence of a functional mammalian nitrate reductase activity also in humans.

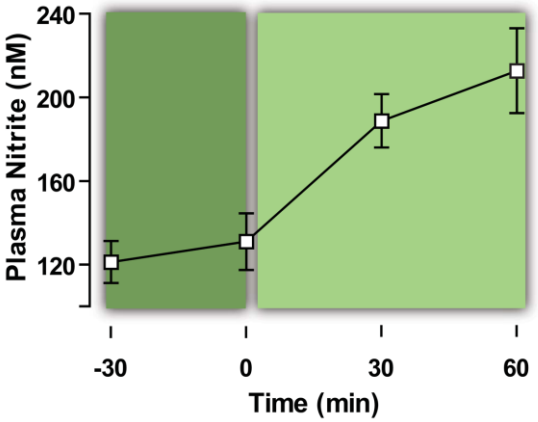


Figure 9. Nitrite levels in human plasma after an oral nitrate load. Plasma nitrite levels were measured repeatedly in 4 healthy subjects before and after an oral intake of sodium nitrate (10 mg kg^{-1}). Before nitrate intake, the subject rinsed the mouth with an antibacterial mouthwash solution (chlorhexidine 2 mg^{-1}) to exclude any contribution from oral nitrate reducing bacteria. Data represent means ± SEM of n = 4. **P* < 0.05 compared to nitrate intake before.

Next, we compared plasma and tissue levels of nitrate and nitrite after nitrate administration in germ-free animals (Paper III). Mice were given nitrate intraperitoneally and levels of nitrate and nitrite were measured in plasma as well as in liver and kidney tissues after 1.5-2 h. In germ-free mice the nitrite levels in plasma, as well as in liver and kidney tissues, increased significantly (Figure 10 a, b) and this increase of nitrite in plasma was attenuated when mice were pretreated with the XOR inhibitor allopurinol (Figure 10 c). This provides final unequivocal evidence for a mammalian nitrate reductase, which is independent on bacteria.

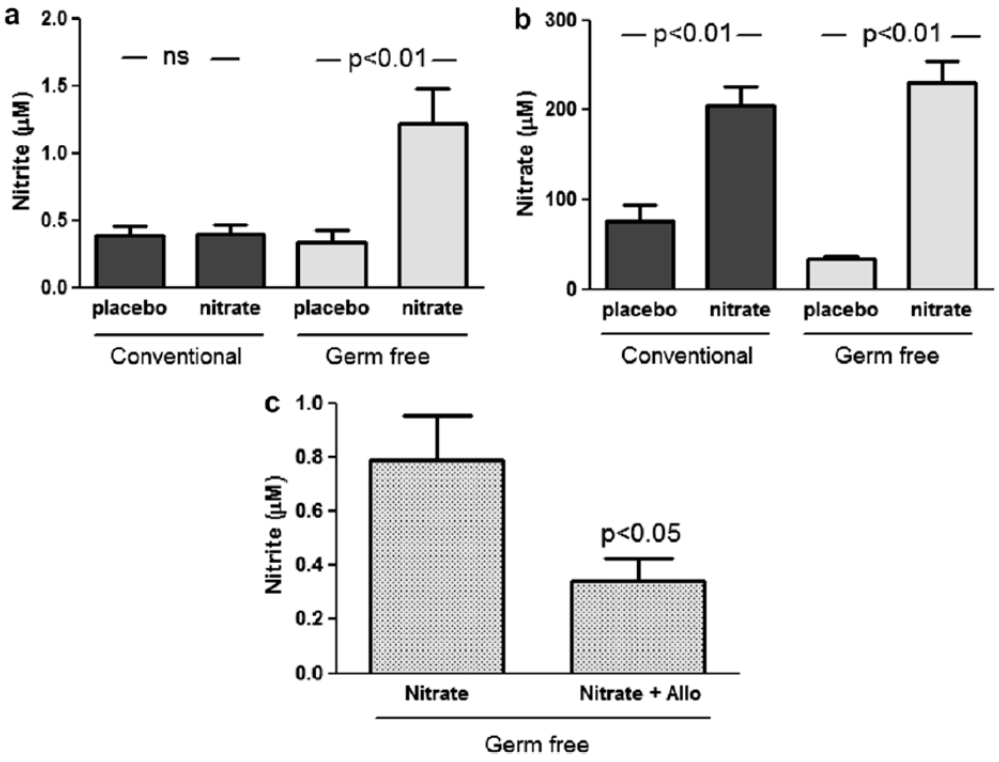


Figure 10. Nitrate reductase activity is enhanced in germ free mice *in vivo*. Plasma levels of nitrite (a) and nitrate (b) were measured 90-120 min after i.p. administration of sodium nitrate (10 mg kg^{-1}) or placebo (NaCl) in germ free and conventional mice. (c) The increase in plasma nitrite was attenuated in germ free mice pretreated with allopurinol (100 mg kg^{-1} day^{-1}).

5.3 XOR- MEDIATED REDUCTION OF NITRATE TO NITRITE AND NO (PAPER II)

To investigate whether the nitrite generated from nitrate is further reduced to NO, we used two different approaches. First, we incubated mouse liver homogenates with nitrite and we found that the nitrite was consumed over the observation period of time and this consumption was inhibited by allopurinol indicating that XOR was involved also in this reaction. Then we added the NO marker DAF-2 DA to mouse liver homogenates and incubated aerobically with nitrite and allopurinol, and analyzed the fluorescent signal indicative of NO generation. A dose dependent increase of the signal was shown but in the presence of allopurinol the signal was inhibited already under basal levels (no nitrate and nitrite added) (Figure 11 a, b). This suggests that XOR

catalyzes a serial reduction of nitrate to nitrite and then to NO in liver tissue under normoxic conditions.

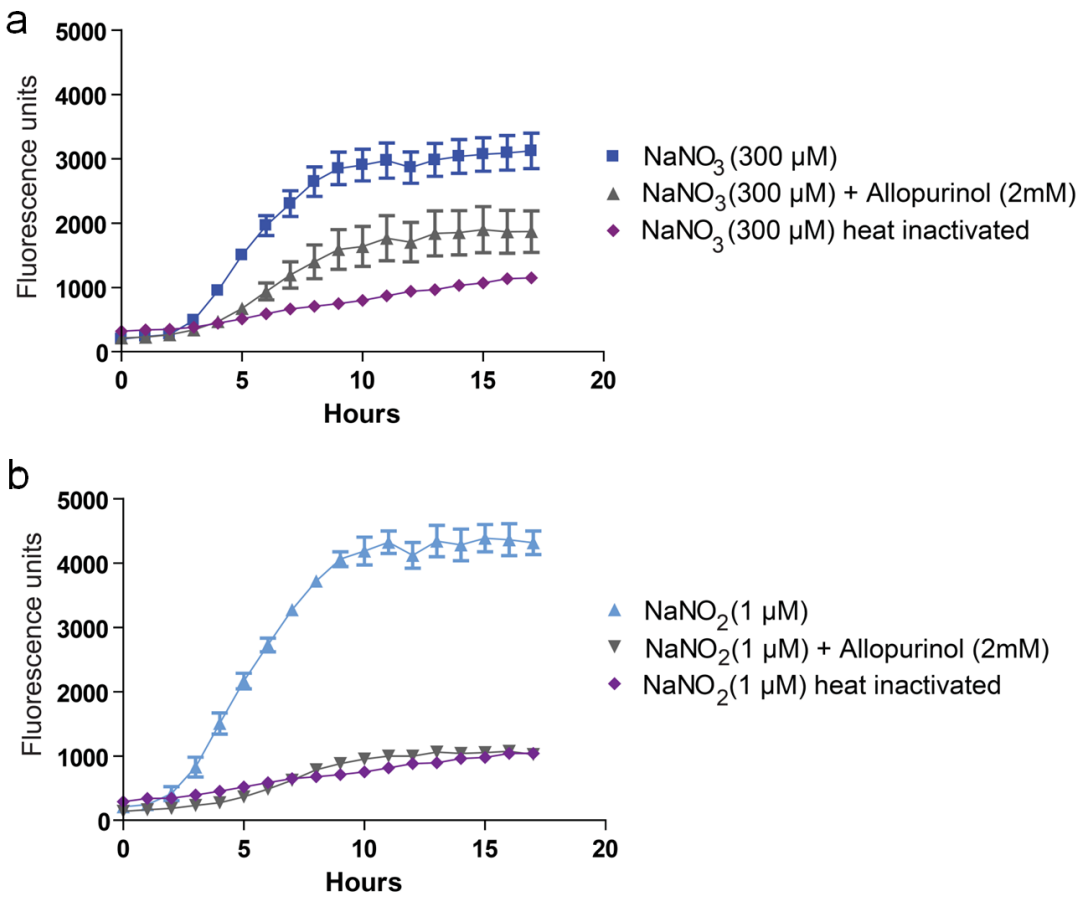


Figure 11. XOR catalyzes a serial of reduction of nitrate to nitrite and then to NO. NO generation was recorded by adding DAF-2 DA (10 µM) to the mouse live homogenates under 15 h aerobic incubation with 300 µM $NaNO_3$ (a) or $NaNO_2$ (1µM) (b). The NO generation was inhibited by allopurinol (2mM) and by heat inactivation of the liver homogenates before incubation.

5.4 EFFECTS OF NITRATE ADMINISTRATION ON BLOOD FLOW AND BLOOD PRESSURE (PAPER II, IV)

In recent studies it has been shown that nitrite infusions at near physiological levels vasodilate the human circulation[23,71] and that dietary supplementation with nitrate causes a reduction in blood pressure[72,73]. To test whether nitrate reduction to nitrite and NO influences post ischemic perfusion (Paper II), we performed a suprarenal clamping of the abdominal aorta in rats for 30 min and then removed the clamp, and monitored blood flow. We found that rats with nitrate treatment maintained a higher post-ischemic blood flow compared to placebo rats. The blood flow decreased to 25% of pre-ischemic values in the control group at 60 min after reperfusion, whereas the rats treated with nitrate maintained blood flow at 65% of pre-ischemic values (Figure 12). These results indicate that the nitrate-nitrite-NO pathway is activated during ischemia-reperfusion injury to maintain blood flow.

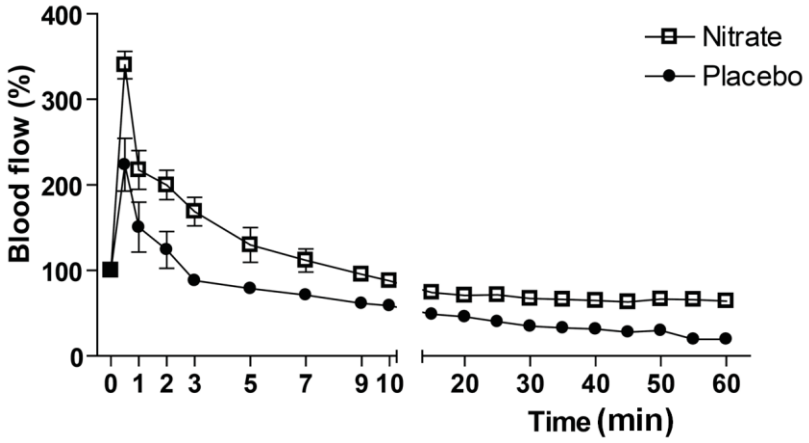


Figure 12. The nitrate-nitrite-NO pathway is activated during ischemia/reperfusion injury. Rats treated with an intravenous bolus dose of sodium nitrate (10 mg kg^{-1}) maintained a higher abdominal aortic blood flow compared to placebo after a suprarenal clamping of the abdominal aorta for 30 min followed by 60 min reperfusion. The clamp was released at time 0.

To examine the effect on blood pressure after a long-term oral nitrate supplementation (Paper IV), we used telemetric measurements. Blood pressure was monitored for 3 days during nitrate supplementation and continuously for 2 days after an acute termination of the nitrate administration. The mean blood pressure was 5 mmHg lower in rats treated with the low dose of nitrate (0.1 mmol kg^{-1} day^{-1}), whereas it was 15 mmHg higher with the high dose (1 mmol kg^{-1} day^{-1}) compared to the control animals (Figure 13 A).

After abrupt termination of nitrate the blood pressure increased by 4 mmHg at day 1 in the animals treated with the high dose nitrate and returned towards basal levels on day 3 (Figure 13 B). In the group of animals treated with the NOS inhibitor L-NAME the acute increase in blood pressure was attenuated in animals treated with nitrate compared to placebo. Collectively, these data suggest that long-term nitrate supplementation down-regulates vascular eNOS activity.

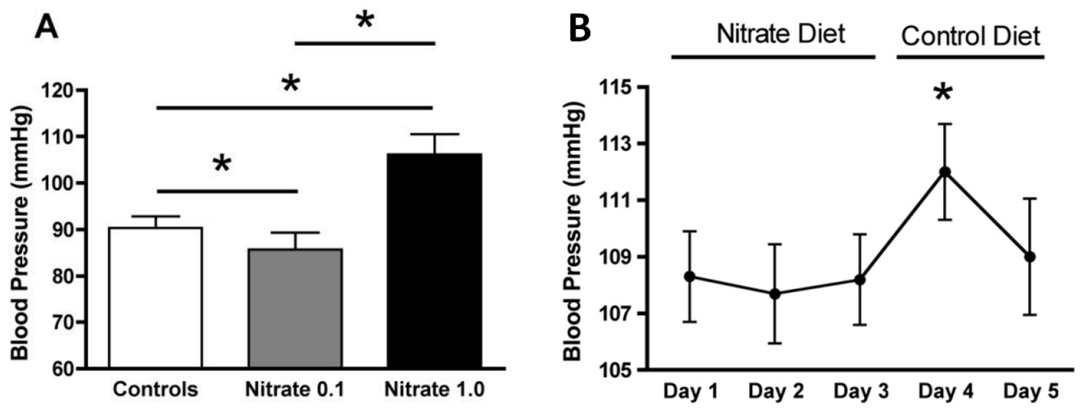


Figure 13. Long-term dietary nitrate supplementation and blood pressure. (a) The mean blood pressure was 5 mmHg lower in rats supplemented with a low dose of sodium nitrate (0.1 mmol kg^{-1} day^{1}), whereas blood pressure was 15 mmHg higher in the group treated with a high dose of sodium nitrate (1 mmol kg^{-1} day^{-1}). (b) After abrupt termination of dietary nitrate, blood pressure in high dose nitrate-treated rats increased by 4 mmHg (day 4) and returned to pre cessation levels at day 5. Data represent means ± SEM of n = 8. **P* < 0.05 compared to controls or nitrate diet.

5.5 PROTEIN EXPRESSION IN GERM-FREE ANIMALS AND THE EFFECTS OF NITRATE (PAPER III, IV)

To study XOR protein expression in germ-free mice (Paper III), we used Western blots. In livers of germ-free mice, XOR expression was significantly increased compared to conventional animals (Figure 14 A, B).

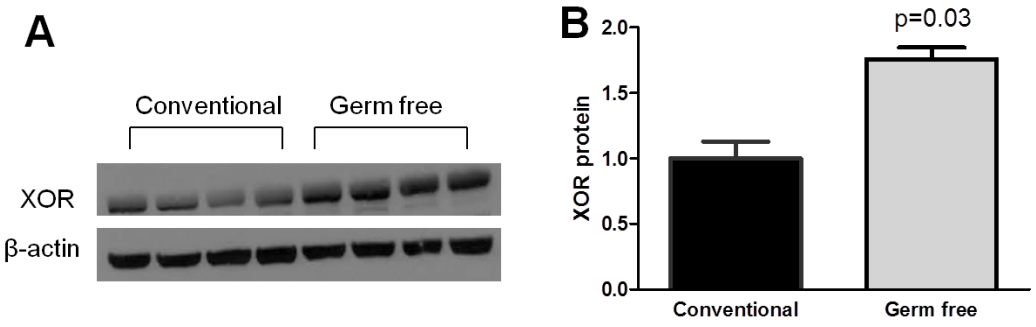


Figure 14. XOR expression is enhanced in germ free mice. XOR protein expression in mouse liver was analyzed by Western blotting. Data represent means ± SEM of n = 4.

To study the effects of nitrate supplementation on eNOS expression (Paper IV), we analyzed eNOS levels in aortas of rats supplemented with nitrate for 8-11 weeks. The expression of phosphorylated eNOS (Ser^{1177}) was dose-dependently reduced by nitrate (Figure 15 A, B).

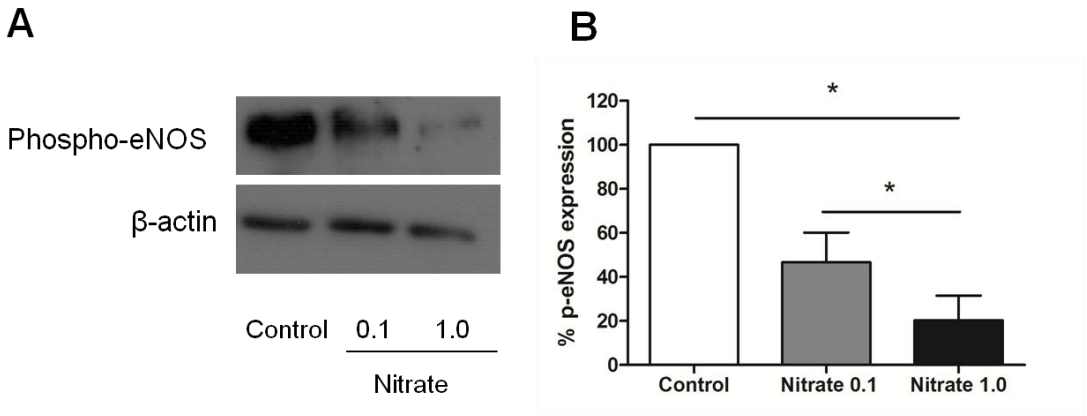


Figure 15. Expression of phosphorylated eNOS is dose-dependently reduced by nitrate. Phosphorylated eNOS (Ser^{1177}, phospho-eNOS) protein expression in rat aortas supplemented with sodium nitrate (0.1 or 1mmol kg^{-1} day^{-1}) for 8-11 weeks was analyzed by Western blotting. Data represent means ± SEM of n = 4. *P < 0.05 compared to controls or between nitrate groups.

5.6 CITRULLINE-ARGININE RATIOS IN PLASMA (PAPER IV)

We measured citrulline to arginine ratio in plasma after the long-term nitrate treatment in rats by HPLC as an indicator of overall vascular NOS activity (Table 1). The citrulline-arginine ratio was significantly decreased in the group treated with a high dose of nitrate suggesting that NOS activity was reduced after the nitrate treatment.

	Arginine *(μM)*	Citrulline *(μM)*	Ornithine *(μM)*	Cit/Arg	Orn/Cit
Controls	153.8 ± 8.6	75.3 ± 1.8	56.1 ± 8.5	0.497 ± 0.027	0.765 ± 0.126
Nitrate 0.1	148.6 ± 8.2	66.6 ± 2.0*	54.3 ± 4.8	0.455 ± 0.021	0.825 ± 0.084
Nitrate 1.0	160.7 ± 7.7	67.1 ± 3.9	68.7 ± 7.6	0.419 ± 0.018*	0.925 ± 0.042

Table 1. Citrulline to arginine ratio is decreased in rats supplemented with a high dose of nitrate. Data represent means ± SEM of n = 8. *P < 0.05 compared to controls.

6 GENERAL DISCUSSION

Eukaryotic cells developed long after the prokaryotes during evolution and these two cell types have been forced to co-exist ever since. The human body is no exception to this and in fact the majority of cells in an adult human are prokaryotes. While pathogens are clearly unwanted and therefore constantly fought, other bacteria exist in peaceful harmony with the host. When such co-existence is of mutual benefit for both parties the relationship is known as symbiotic. Microbes that reside in our body are primarily located in the gastrointestinal tract, with the highest density in the oral cavity and the large intestine.

The interplay between the host and some of these gut bacteria is clearly symbiotic with benefits for the host in maintaining integrity of a variety of mammalian processes, including metabolism, immune defense, inflammatory responses, nutrient processing and a broad range of other host activities[74-76]. In sharp contrast to this, the generation of nitrite by some gut bacteria has traditionally been viewed as harmful with formation of carcinogenic nitrosamines as a possible result[77]. This view is slowly changing and we may now be close to a paradigm shift in our view of nitrogen oxide metabolism in humans[5,33,78].

The results presented in this thesis add to this growing body of evidence suggesting a symbiotic rather than pathological relationship between nitrate-reducing gut bacteria and the host. Gut bacteria help the host to bioactivate inorganic nitrate to nitrite and then to nitric oxide, a powerful signaling molecule with numerous beneficial effects in the gut, cardiovascular system and elsewhere. In return they are provided with a substrate necessary for their own respiration. In addition, this thesis describes a previously unknown pathway for the reduction of nitrate by mammalian cells that may contribute to tissue NO homeostasis. Finally, we put forward evidence to suggest a crosstalk between eukaryotic and prokaryotic NO pathways within the human body. Central to all this - and representing the first obligate step in bioactivation - is the initial reduction of nitrate to form the more reactive nitrite anion.

6.1 PROKARYOTIC NITRATE REDUCTION AND NO FORMATION IN THE GUT

Bacterial nitrate reduction plays a critical role in the bioactivation of nitrate in humans. The nitrite formed by oral bacteria can enter the systematic circulation and be further reduced to NO by a variety of non-enzymatic and enzymatic processes in our body[7,42]. Recent studies have shown that if the oral microflora is abolished with the use of an antiseptic mouthwash, nitrite formation is attenuated and the resultant acute biological effects, including a reduction in blood pressure, are reduced or absent[79]. Moreover, if saliva is not continually swallowed after ingestion of nitrate, thereby interrupting the entero-salivary circulation of nitrate, the increase in plasma nitrite is attenuated as are the biological effects[39,80]. Altogether, this demonstrates a central role of oral bacteria in bioactivation of nitrate (Figure 16).

While these interesting effects of oral bacteria have been fairly well characterized recently, along with the NO formation and biological effects of nitrite-derived NO in the stomach, less is known about nitrate and nitrite metabolism further down in the GI tract. An aim of the present thesis was to study the metabolic fate of nitrate in the small and large intestine. Specifically we analyzed if NO could be generated by probiotic bacteria. Indeed, dietary supplementation with live probiotic bacteria and nitrate enhanced NO formation locally in the gut. Interestingly, some bacteria in the gut can also effectively consume NO formed by other microbes. The reduction of nitrite to NO by probiotic bacteria is likely a result of their ability to reduce pH by production of lactic acid as demonstrated in our in vitro experiments. Under such conditions nitrite is effectively reduced non-enzymatically to form NO. In addition, an enzymatic component, for example by the action of bacteria nitrite reductases, cannot be excluded at this stage.

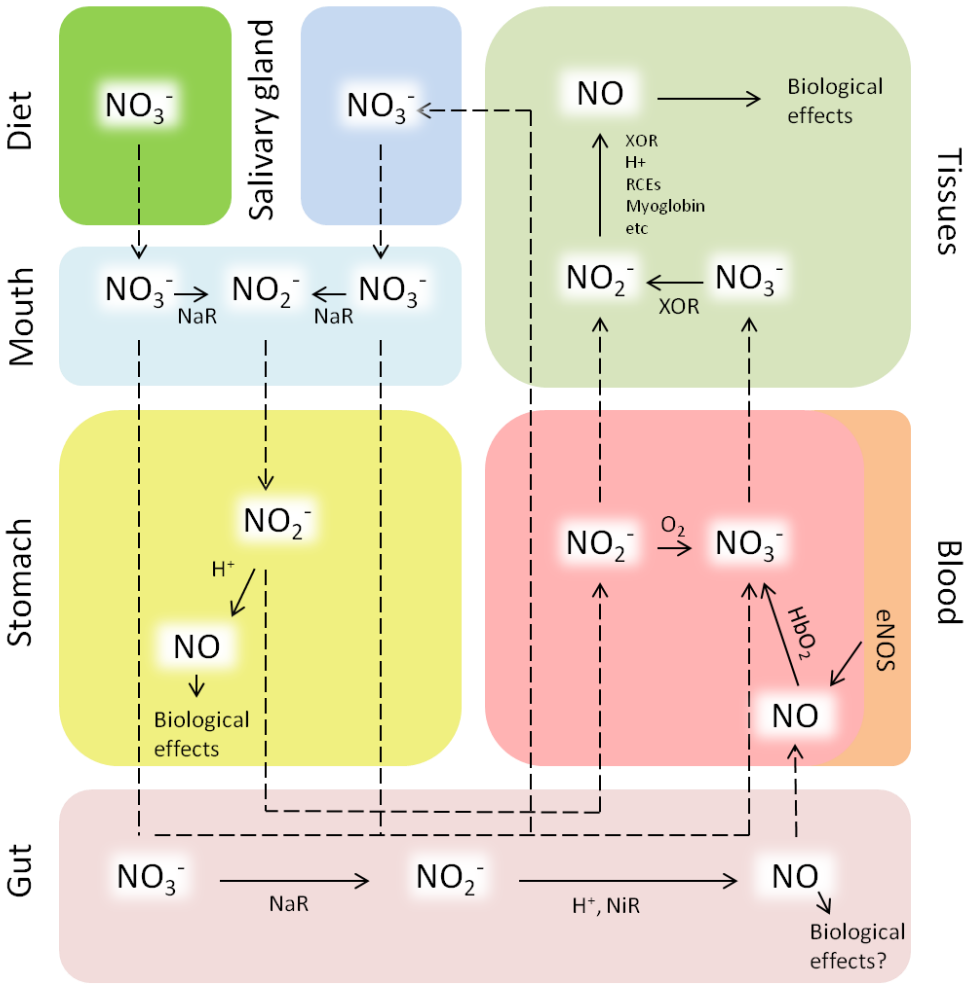
The proposed beneficial health effects of probiotics have been intensively studied and although definitive proof is still lacking for most of these claims, it is clear that millions of people throughout the world consume such live bacteria on a daily basis in the form of beverages, yoghurts and more. The possible biological consequences of bacterial NO formation in the gut and its relation to any health effects of probiotics, can only be speculated upon at this stage. It is interesting however to note that the levels of NO obtained are clearly within the range where this potent messenger is active. Effects of NO and other nitrogen oxides produced by bacteria may include inhibition of pathogen growth but also effects on the host mucosa including stimulation of mucosal blood flow and mucus generation. Such effects of nitrite-derived NO have already been demonstrated in the stomach but evidence for the lower GI tract is still missing. In addition to effects on blood flow and mucus formation, nitrite and NO can also affect other processes in the cell. Such effects include potent cytoprotective actions and a possible target is the mitochondrion with inhibition of reactive oxygen species formation[81,82].

6.2 MAMMALIAN NITRATE REDUCTION TO FORM NITRITE AND NO

A surprising finding in the present thesis was the fact that mammalian cells are also capable of nitrate reduction (Figure 16); a chemical reaction previously thought to be performed exclusively by anaerobic bacteria. From biochemical and pharmacological studies in rodents and humans we demonstrate that xanthine oxidoreductase, an enzyme structurally related to bacterial nitrate reductase, is a functional mammalian nitrate reductase. Clearly mammalian nitrate reduction is much less effective than bacterial nitrate reduction. Overall however, the relatively inefficient nitrate and nitrite reduction by mammalian cells might still be significant, especially in close vicinity to the mammalian nitrate reductase. Thus, micromolar nitrate yields high nanomolar nitrite and eventually picomolar NO in the target tissue, still enough to elicit powerful biological effects. Indeed, in the current thesis we demonstrate increases in post-ischemic blood flow in animals treated with nitrate i.v. Such an effect is likely attributed to NO formation from the serial reduction of nitrate although definite proof for this was not obtained from the current study. Nevertheless, the true biological significance of mammalian nitrate reduction remains to be elucidated.

A specific remaining overall question in this field is if the normal levels of nitrate and nitrite generated endogenously by the NOS system are sufficient for biological effects? This occurrence would demonstrate a true physiological role for the nitrate-nitrite-NO pathway.

Most studies so far have looked at administration of exogenous nitrate/nitrite, using either pharmacological doses or amounts achieved by dietary intake of these anions. The relative importance of mammalian nitrate reduction in relation to that performed by bacteria in the oral cavity is also an unresolved issue. As discussed above many of the NO-like effects of nitrate described, including effects on blood pressure and gastric protection, seem to be almost abolished if oral bacteria are killed. Does this leave any room for mammalian nitrate reduction to be of any significance? It is possible that the mammalian nitrate reduction is of greater significance in rodents compared to humans as these animals have much greater expression of XOR throughout most tissues. Another possibility is that mammalian nitrate reduction is a slower system meaning that a longer observation time is needed before the biological effects can be observed. Future long-term studies of nitrate effects in germ-free animals will definitely answer this last question. Such animals offer the unique possibility to study exclusive mammalian processes.



Diet	- The diet is a main source of inorganic nitrate with vegetables being particularly rich in this anion
Salivary gland	- The salivary glands actively concentrate dietary and endogenously produced nitrate from plasma
Mouth	- Salivary nitrate is partly reduced to nitrite by nitrate reductase (NaR) in oral commensal bacteria
Stomach	- Nitrite forms nitric oxide and other bioactive nitrogen oxides non-enzymatically in the acidic stomach
Gut	- The majority of remaining nitrate and nitrite is absorbed in the small intestine and enters the blood - Bacteria generate NO locally in the gut lumen via serial reductions of remaining nitrate and nitrite
Blood	- In blood nitrate, nitrite and NO mixes with NO coming from the endogenous NO synthase pathway - Oxidation of nitrite and NO leads to rapid formation of nitrate in blood
Tissues	- Xanthine oxidase (XOR) in tissues generates small amounts of nitrite from nitrate via reduction - Various enzymes can catalyze further reduction of nitrite to bioactive NO

Figure 16. Scheme showing a mammalian nitrogen oxide cycle in which inorganic nitrate, nitrite and NO are interconverted *in vivo*. In this cycle nitrate and nitrite from endogenous and dietary sources are serially reduced by bacteria as well as mammalian cells to form NO and other bioactive nitrogen oxides.

6.3 INTERACTIONS BETWEEN NOS-INDEPENDENT AND NOS-DEPENDENT NO PATHWAYS

Generation of NO in a mammal is a tightly regulated system, which is controlled in most situations by negative feedback and feedforward controls. If the newly found pathway of NO formation from mammalian nitrate reduction is significant, one would expect that the classic L-arginine-NOS pathway and nitrate-nitrite-NO pathway could communicate with each other to orchestrate NO formation, metabolism and bioavailability. Studies from this thesis support this view (Figure 17). Long-term dietary nitrate supplementation results in not only down-regulation of eNOS protein expression but also decreased eNOS activity. Cardiovascular NO homeostasis is predominantly regulated by eNOS. It is therefore perhaps not surprising that NO generation from mammalian nitrate reduction influence synthesis and catabolism of NO in the cardiovascular system. This could then be reflected in changes in blood pressure, keeping in mind the vasodilating properties of NO. An interesting finding in this thesis is the apparent negative feedback on eNOS by the nitrate pathway, showing that a low physiological dose of nitrate decreased blood pressure, whereas a high pharmacological dose of nitrate paradoxically elevated blood pressure. It seems that when the nitrate-nitrite-NO pathway is maximally boosted, the net effect is a reduced NO bioavailability since the inhibition of eNOS overrides the NO formation from nitrate. Importantly however, this was done in young healthy rats with a well-functioning highly active eNOS. Thus, inhibition of eNOS will have a dramatic effect. In a situation where eNOS activity in the endothelium is diminished, such as in older subjects or in patients with cardiovascular diseases, the effects of nitrate are expected to be different. In this case eNOS inhibition has a minor effect (there is not much eNOS to inhibit) so that the net effect of NO from the nitrate instead becomes significant. This is strongly supported by a recent study, in which we gave a high pharmacological dose of nitrate to rats with hypertension and decreased NO bioavailability. In these rats we observed a dramatic 24 mmHg reduction in blood pressure after nitrate[83]. All together, we speculate that administration of nitrate will likely have a more obvious and beneficial effect on NO homeostasis in individuals where eNOS is not functioning properly. If this is correct, it could have a major impact on future dietary recommendations.

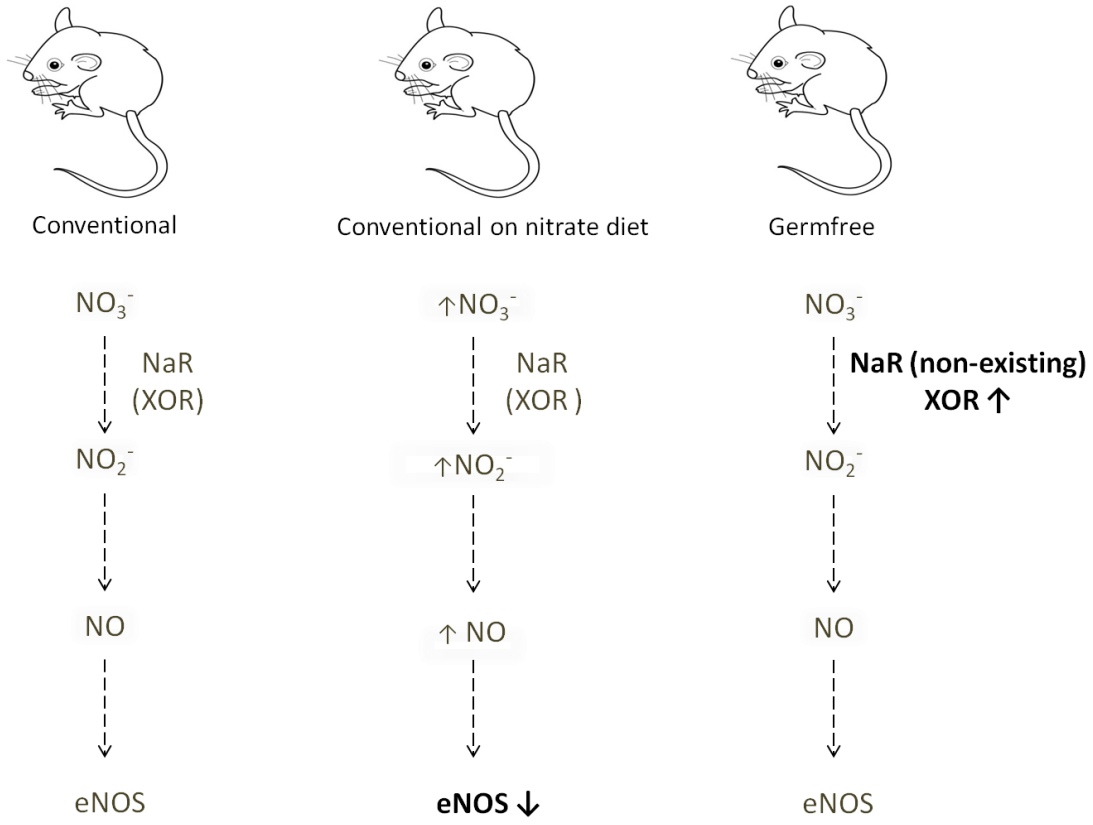


Figure 17. Scheme illustrating sites of a crosstalk between the nitrate-nitrite-NO pathway and the classical NO synthase (NOS) pathway. The enzymes affected are highlighted. When conventional mice are fed nitrate a downregulation of eNOS in the vascular system is observed, likely as a result of increased NO formation via the nitrate-nitrite pathway. By definition germfree animals cannot reduce nitrate via prokaryotic pathways (NaR). In these animals the expression of XOR, a mammalian enzyme with nitrate reductase activity is increased in tissues, possibly to maintain a certain degree of nitrate reductase activity in the absence of bacteria.

6.4 THERAPEUTIC PERSPECTIVES

Although the physiological and therapeutic effects of nitrate and nitrite reduction are yet to be fully verified, several lines of studies convincingly indicate therapeutic opportunities of the nitrate-nitrite-NO pathway in various diseases, most notably in the cardiovascular system. Many studies have demonstrated cytoprotective effects of low-dose nitrite in mice, rats, sheep, dogs, rabbits, primates and humans exposed to different degrees of ischemia-reperfusion (IR) injury[84-90]. Nitrite and nitrate administration shows protective effects against IR injury in liver[91,92], heart[87,91,93,94], brain[94], kidney[95], as well as in chronic hind-limb ischemia[96]. These cytoprotective effects, which are unrelated to vasodilation, suggest therapeutic potentials of nitrite and nitrate to treat human diseases associated with ischemia-reperfusion, such as myocardial infarction, stroke, solid-organ transplantation, and cardiopulmonary arrest.

The mechanism for the cytoprotection needs to be clarified but research point towards the mitochondria as an important target[82,97]. Mitochondria is a well-known target of NO effects, and studies have shown that nitrite-derived NO may interact with the respiratory chain enzymes to control respiration and ultimately reduce the generation of potentially damaging reactive oxygen species (ROS).

Therapeutic delivery of high doses of nitrite to vasodilate ischemic vascular beds also shows promise in preclinical studies. Systemic infusions of nitrite to a primate model of artery aneurysm can effectively protect against delayed cerebral artery spasm[89,98]. Inhalation of nitrite in newborn sheep with primary pulmonary hypertension can selectively dilate the pulmonary circulation and improve oxygenation via nitrite reduction to NO[86]. Recent clinical studies also showed that inhalation of NO has protective effects on liver function after orthotopic liver transplantation in humans[99] and on the inflammatory ischemia/reperfusion response after surgery[100]. In these studies, the authors suggested that nitrite (the oxidation product of NO) is the most likely candidate transducing the exptrapulmonary effects of inhaled NO. This is because NO itself is unlikely to survive blood passage to distal organs after inhalation.

Moreover, an increasing number of studies show that supplementation of dietary nitrate or nitrite can reduce blood pressure[72,80,101], protect against gastric ulcers[32], prevent renal and cardiovascular dysfunction induced by a high-salt diet[83], decrease platelet aggregation[102], protect ischemic cardiac tissue[91] and reverse features of metabolic syndrome in mice lacking endothelial NOS[103].

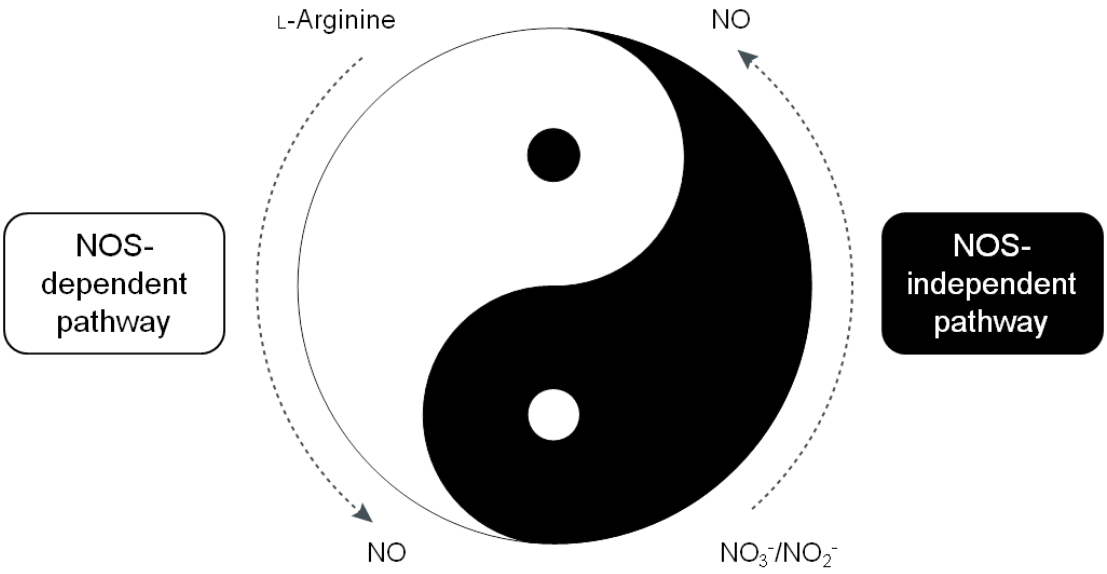
7 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis we have continued to characterize the biological chemistry, physiological effects and therapeutic opportunities of dietary nitrate - an inorganic anion generated endogenously and present in our everyday diet. We show that nitrate can be bioactivated *in vivo* to form nitrite and then bioactive nitrogen oxides including NO. The initial reduction of nitrate to nitrite represents an obligate first step in its bioactivation. We show that commensal bacteria in the GI tract as well as mammalian host cells are capable of nitrate bioactivation. Oral commensal bacteria generate nitrite via highly effective nitrate reductase enzymes, while our own cells use XOR for the same reaction albeit at a much lower efficiency. In the lower GI tract probiotic bacteria can generate NO locally from nitrate-derived nitrite with possible biological effects. The nitrite that is absorbed intact can be utilized by cells to generate NO in the tissues. Interestingly, nitrite generation to NO in the tissues is greatly enhanced during acidic and hypoxic conditions when the classic NO synthase system is malfunctioning. Thus the nitrate-nitrite-NO pathway can be viewed as a reserve system to allow NO generation when NO is needed the most. This is analogous to anaerobic glycolysis as a source of ATP under hypoxic conditions.

The therapeutic opportunities for nitrate and nitrite are promising and larger clinical trials in humans are currently underway. Conditions that might be targeted by these pathways include hypertension, ischemia-reperfusion injury, metabolic disease, infections, vasospasm and gastric ulcers. There are at least four important differences that favor nitrate/nitrite as drug candidates compared to the organic NO donors, such as nitroglycerine that have been in clinical use for several hundred years. These are their relatively low potency, the favorable pharmacokinetic profile, the selective activation in ischemic areas and the lack of tolerance. Nitroglycerine is an extremely potent vasodilator while nitrate and nitrite have relatively low potency. However, for treatment of hypertension, a rapid and short acting vasodilator is not optimal since blood pressure then becomes very difficult to control. Indeed organic nitrates have not found their way into the clinic for this condition. In addition, treatment with continual organic nitrates classically results in tolerance, a condition where repeated dosing leads to a severe decline in the effects. Such tolerance seems to be absent for nitrate and nitrite. Moreover, nitrate has a long t ½ which is desirable in the clinical situation. Lastly, nitrite is selectively bioactivated in ischemic areas, i.e. in areas where NO is needed the most. Thus, nitrite reduction to NO is redox sensitive and accelerated under these conditions. Organic nitrates on the other hand, release the NO unselectively in many tissues.

The dietary implications of this research are particularly intriguing, since the doses of nitrate needed for significant effects are well within what is achievable from a normal diet. The traditional view of nitrate and nitrite is that they are only harmful. The proposed associations with cancer have resulted in strict regulations of the acceptable daily intake of these anions. However, despite 50 year of research into these detrimental effects of nitrate, evidence for a carcinogenic effect in humans is still lacking[104-107]. With the data accumulated during the past 15 years, a new picture is slowly emerging; a picture suggesting the exact opposite: Dietary nitrate may in fact have beneficial effects on human health and the mechanism is via the formation of NO

- a central biological messenger in the cardiovascular system. It is our hope that in some years people will regard dietary nitrate not as a toxic unwanted substance but rather as an essential nutrient. Time will tell if this provocative speculation is true.



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