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DIPLOMARBEIT

THE ROLE OF METHANE IN THE REGULATION OF NITRITE REDUCTASE ACTIVITIES IN ISCHEMIC MAMMALIAN TISSUES

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unter der Anleitung von

a.o. Univ.Prof. Dipl.-Ing. Dr.techn. Heinz Redl
Univ.Doz. Dr. Andrey Kozlov

durch

Tanja Stögerer

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Declaration of Authorship

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Abstract

During ischemia, the formation of the potent vasodilator and gasotransmitter nitric oxide (NO) mainly takes place via a reduction of nitrite by a family of enzymes and structures called nitrite reductases. In the first part of this project, we have characterized the kinetics of nitrite reduction to NO in different organs without being able to explain the differences in the kinetics of their NO release. The second part of the study was aimed at understanding the mechanisms underlying the kinetics of NO release, particularly the origin of an observed lag-phase. In the last part we identified the major generator of NO in the intestine and its regulation by methane, a gaseous messenger recently discovered in mammalians. The release of NO was monitored using a continuous chemiluminescence NO detection method. We determined the overall NO released from tissue homogenates prepared from the liver, small intestine and heart of Sprague Dawley rats under conditions simulating ischemia. The individual contributions of the previously identified nitrite reductases hemoglobin, myoglobin, xanthine oxidoreductase, and cytochrome bc1, a part of the mitochondrial respiratory chain, were then assigned using different inhibitors. Under severe hypoxia and slightly acidic conditions, xanthine reductase was found to be the main contributor to the NO release from nitrite, compared to hemoglobin, myoglobin and cytochrome bc1. This finding was used to investigate a possible interplay between NO and the recently identified gasotransmitter methane (CH_4), which has been shown to decrease the NO-mediated tyrosine nitrosylation in ischemia-reperfusion injury. The ability of CH_4 to decrease NO levels has previously been observed in an *in vivo* study. In this study, rats undergoing ischemia and reperfusion in the intestine were ventilated with air containing 2.2 % CH_4, exhibited significantly reduced tissue damage and release of inflammatory mediators, such as NO. This could be replicated *in vitro*, as the NO release from rat intestine homogenates was significantly decreased in measurements using a mixture of 2.2 % CH_4 in nitrogen as a carrier gas, as compared to the control measurements under N_2. To clarify the mechanism of this phenomenon, we performed *in vitro* experiments. First, we examined the effect of CH_4 on the NO production from pure xanthine oxidoreductase, supplying only electron donors acting at the molybdenum site of the enzyme, i.e. xanthine and hypoxanthine. In this NO release from xanthine and hypoxanthine conversion by xanthine oxidoreductase, the presence of CH_4 had a contrasting effect, increasing rather than decreasing the NO measured. Similarly, xanthine oxidoreductase supplied with a single electron donor, NADH, which acts at the FAD site of the enzyme, gave rise to an increase in NO production in the presence of CH_4; however, this effect was counteracted by the addition of the additional electron acceptors and other enzymes using NADH as a cofactor. By boiling the homogenate, we inactivated all enzymes while maintaining physiological concentrations of the substrates NADH, NAD^+, xanthine and hypoxanthine. To limit thermal decomposition of the substrates, the duration of boiling was set to 5 minutes, at which point still about 75 % of NAD^+, the least stable of the compounds, remains. In these experiments, exogenous xanthine oxidase reproduced the effect observed *in vivo*, where CH_4 inhibited NO generation. DPI, an inhibitor of the FAD site of the xanthine oxidase enzyme, which blocks the interaction with NAD^+ and NADH, reversed the effect of

CH_4 on NO generation. This opposing CH_4-induced effect might be attributed to an increased incorporation of NO in tissues (e.g. protein nitrosylation) which results in less free NO in the medium, a process which is represented as a lag-phase in NO release from homogenate samples.

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Table of Contents

1. Introduction

1.1. Ischemia-reperfusion and hypoxia-reoxygenation

Ischemia-reperfusion injury is a form of cellular damage caused by a two-step clinical process consisting of a period of restricted blood flow in a tissue, called the ischemic phase, followed by a reconstitution of blood circulation, named reperfusion phase. This might occur naturally in pathological settings such as hemorrhagic shock, stroke and myocardial infarction, as well as during clinical procedures, e.g. organ transplantation and cardiopulmonary bypass surgery.

Under ischemia, the restricted blood flow causes a shortage of oxygen needed for various essential biological processes, not the least of which is the production of energy via the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This lack of energy causes the cells to switch to an anaerobic metabolism, which does not rely on oxygen but leads to a locally increased concentration of lactic acid, a by-product of the anaerobic energy production. This resulting significant decrease in pH in the ischemic tissue is called metabolic acidosis. With depleting ATP storage, the cells lack sufficient energy to maintain homeostasis, which eventually results in a loss of cell membrane potential.[1]

During reperfusion, the restoration of blood flow in the tissue causes reintroduction of oxygen to the cells. Any built-up metabolites are flushed from the local site of ischemia and introduced to the systemic circulation. Further injury to the cells and tissue, such as ischemia-reperfusion injury and local and systemic inflammation, is caused by highly reactive oxygen-derived free radicals (Figure 1). Both systemic inflammatory response syndrome (SIRS), a condition similar to sepsis from bacterial infection, and multiple organ dysfunction syndrome (MODS), during which altered organ function is not able to upkeep homeostasis without any clinical intervention[2], can occur in severe cases. It is thereby one of the leading causes of death in intensive care patients.[1,3]

Not unlike ischemia-reperfusion, hypoxia-reoxygenation consists of a phase of oxygen deprivation in a tissue, termed hypoxia phase, and a second period in which sufficient oxygen is re-introduced to the tissue, called reoxygenation. Oxygen concentrations during the hypoxic phase can range from anoxia, which is the complete absence of oxygen, to severe hypoxia, meaning a lack of oxygen relative to the biological demand. Ischemia-reperfusion and hypoxia-reoxygenation are sometimes used synonymously, however, while blood flow is obstructed during the ischemic phase in ischemia-reperfusion, leading to a local build-up of harmful by-products of anaerobic energy generation, no change in blood flow is observed under hypoxia-reoxygenation. Conditions under which hypoxia-reoxygenation can occur include shock, trauma, exposure to high altitudes and a number of other pathological situations.[4] Despite still maintaining blood flow during hypoxia as opposed to the restricted circulation in ischemia, it has been shown in rat liver that hypoxia/reoxygenation is as damaging to the tissue as ischemia/reperfusion. [5]

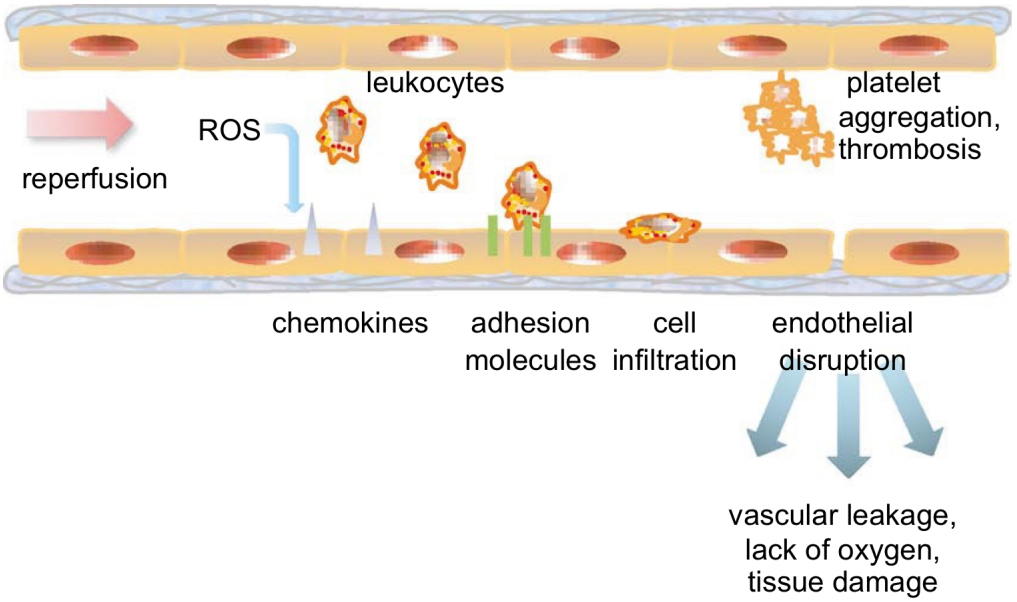


Figure 1 Pathological processes causing tissue damage upon ischemia-reperfusion.
After ischemia, reperfusion restores oxygen levels in ischemic tissues, thereby generating a number of reactive oxygen species (ROS). These ROS give rise to oxidative stress and cause damage to cells and organs. Furthermore, ROS induce an increased production of chemokines on the endothelial wall. All this leads to a disruption of the endothelium, which then causes vascular leakage and further damage by infiltration of leukocytes in the tissue. Adapted from [6]

The harmful imbalance of oxidants and antioxidants in favor of oxidants leading to ischemia-reperfusion injury is named oxidative stress.[7] If this excess of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is not counterbalanced by defense mechanisms like oxygen radical scavenging enzymes, e.g. superoxide dismutase, or antioxidants like tocopherol, it can lead to damage to DNA, lipids or proteins. This damage can kill the cells or induce their carcinogenic transformation.[8]

The reactive oxygen species causing oxidative stress can occur either as a reactive molecule or as free radicals, which are defined as unstable molecules containing at least one unpaired electron, making them highly reactive. Analogously, the reactive nitrogen species involved in oxidative stress are either in the form of free radicals, or a highly reactive molecule or ion.[9]

An important free radical reactive oxygen species is superoxide ($O_2^{\cdot -}$), which is a negatively charged byproduct of various cellular processes such as mitochondrial respiration, and electron transport across membranes, and also produced by a number of soluble proteins such as xanthine oxidoreductase, hemoglobin and aldehyde oxygenase. In comparison, hydrogen peroxide (H_2O_2) is a less reactive member of ROS, which also occurs as a product in normal physiological processes.

The main reactive nitrogen species occurring in the body is nitric oxide (NO). This endogenously produced gaseous molecule is vital for many biological processes, such as controlling vasodilation via cyclic guanosine monophosphate (cGMP), a second messenger molecule. An excess of NO, however, can have deleterious effects to cells and tissue and can lead to increased oxidation to peroxynitrite, which, while being mentioned to be involved in cell signaling processes, can also lead to lipid peroxidation and which causes severe cellular damage.

1.2. Properties and Pathways of NO

As the first discovered gasotransmitter, nitric oxide was originally termed endothelium-derived relaxing factor before its nature was completely uncovered, ultimately earning its discoverers Robert F. Furchgott, Louis J. Ignarro and Ferid Murad the Nobel Prize in Physiology and Medicine in 1998. This was an exciting discovery not only because NO was not known to be endogenously produced in mammals, but also because it was not suspected to have any biological function.

Today, NO is known to be synthesized in the body at physiological concentrations of about 100 pM to 5 nM and has been shown to be involved in various biological processes shown in Figure 2.[10] Besides regulating the vascular tone by stimulating guanylate cyclase to produce cGMP as well as playing a part in the stimulation of angiogenesis, nitric oxide was found to inhibit aggregation of both leukocytes and platelets and has a cytoprotective effect against ischemia.[11–14] On the other hand, at elevated levels and under pathological conditions, NO has a detrimental effect by aggravating oxidative stress, inhibiting mitochondrial respiration and inducing apoptosis.[15,16]

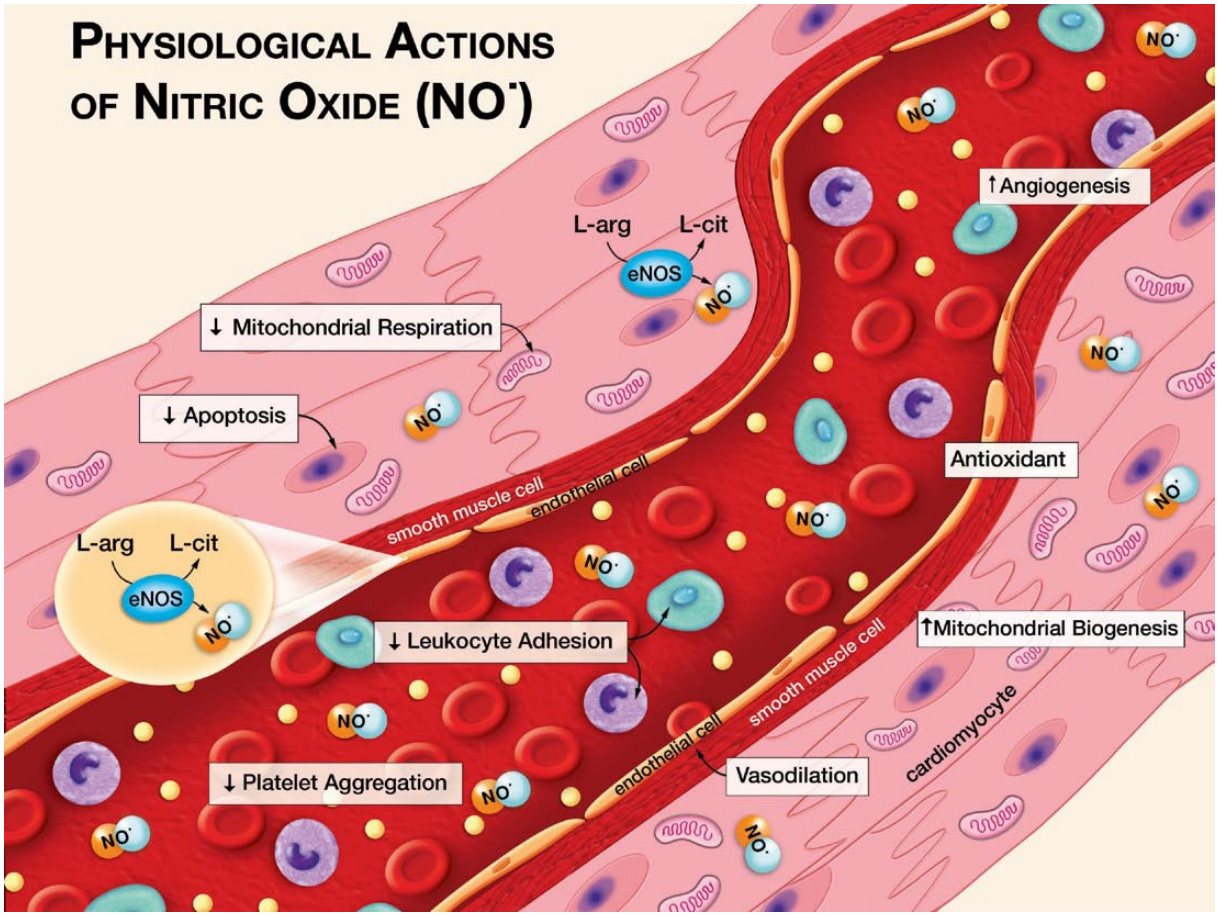


Figure 2 Physiological actions of NO.
Nitric oxide takes part in many different biological processes. At physiological levels, NO regulates vasodilation, inhibits mitochondrial respiration, apoptosis, and aggregation of platelets and leukocytes. It also increases mitochondrial biogenesis and angiogenesis. Adapted from [17]

Under normal physiological conditions, the majority of NO is produced via a group of enzymes called Nitric Oxide Synthases (NOSs). They exist as three isoforms, two of which

are constitutively expressed, e.g. the neuronal Nitric Oxide Synthase (nNOS) and the endothelial Nitric Oxide Synthase (eNOS). They produce NO in calmodulin-regulated short bursts. Another form of NOS is the inducible Nitric Oxide Synthase (iNOS), which has a calmodulin subunit and can, therefore, produce NO over a longer period of time in a state of permanent activation.[18,19]

For all NOS isoforms, NO is produced by a reduction of L-arginine to L-citrulline in an enzymatic reaction which is dependent on a number of cofactors, such as NADPH, FMN, BH_4, and FAD, as well as the substrates L-arginine and oxygen. In the presence of insufficient amounts of oxygen, enzymatic functions are drastically altered for all three isoforms, however, they have different oxygen requirements with K_M values ranging from 23 µM for eNOS to 350 µM for nNOS.[19]

In a hypoxic setting, NO formation switches from the strongly oxygen-dependent NOS pathway to alternative ways of NO generation, which are more suited to the conditions, such as the production of NO from nitrite reduction (Figure 3). Nitrite, which has long been viewed as an inert oxidative breakdown product of NO, is now known to be a critical functional storage form of NO, which can be used for NO formation under pathological conditions that are unfavorable for NOS activity.[20,21] This generation of NO from nitrite provides crucial tissue and cytoprotective effects in ischemia/reperfusion models and has been shown to have tissue protective effects on the vasculature and the myocardium.[22,23]

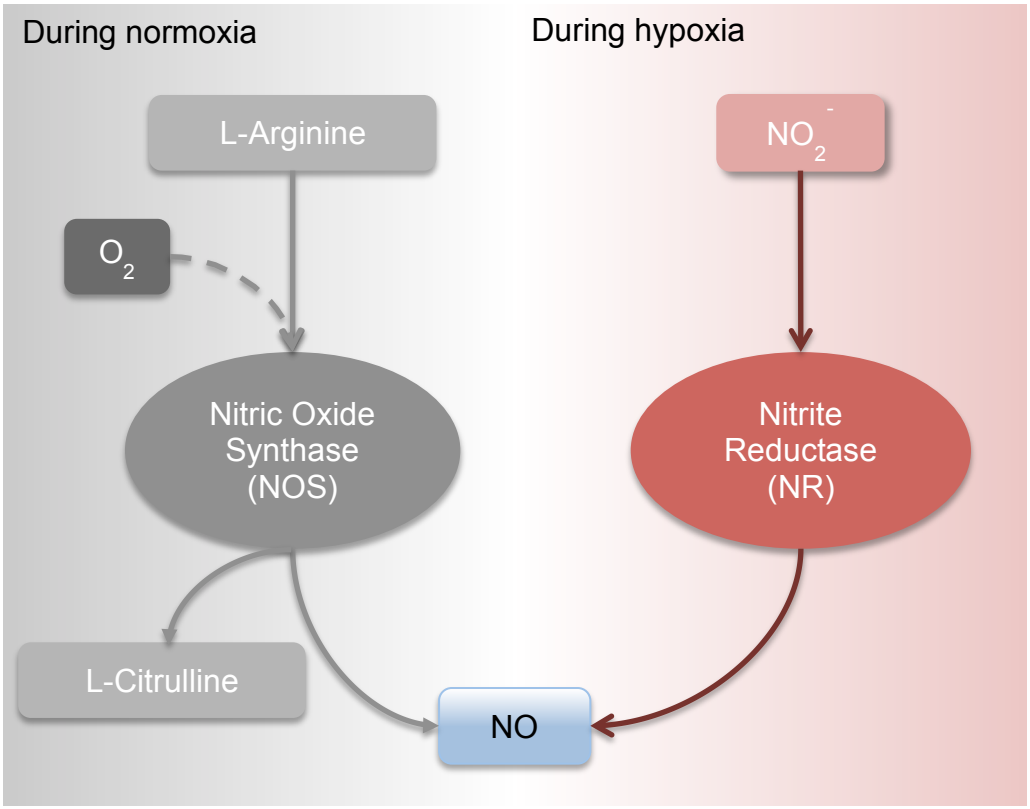


Figure 3 Pathways of NO formation under physiological conditions and hypoxia
Under physiological oxygen tension in the tissues (normoxia), nitric oxide is predominantly produced by the nitric oxide synthases, eNOS, nNOS, and iNOS. Due to the lack of sufficient oxygen levels, NO formation switches to an anaerobic reduction of nitrite by various nitrite reductases, such as hemoglobin, myoglobin, xanthine oxidoreductase, and mitochondria under hypoxic conditions.

A number of structures and enzymes have been identified as capable of this nitrite reduction, and consequently grouped together under the name nitrite reductases (NRs). These NRs include mitochondria, or more specifically a part of the mitochondrial respiratory chain, and metalloproteins such the iron-centered hemoglobin and myoglobin, and xanthine oxidoreductase, which carries Molybdenum as its active center.

Mitochondria, while being inhibited at the cytochrome c oxidase by excess levels of NO,24 have been suggested to contribute to NO production both aerobically, using a Ca^{2+}-dependent type of nitric oxide synthase located on the mitochondria (mtNOS),[25] as well as under anaerobic conditions. However, the existence of mtNOS is questionable.[26]

Their nitrite reductase activity has been attributed to an interaction of ubisemiquinone with cytochrome bc1 and was shown to be inhibited by myxothiazol.[27]

The tetrameric protein hemoglobin consists of two alpha- and two beta-subunits, each of which are centered around a heme group, an iron-containing prosthetic group. It conducts its chief role, the transport of oxygen through the blood system, by transitioning from a so-called T-state of low oxygen affinity, during which hemoglobin is fully deoxygenated, to a state of high oxygen affinity, the R-state, by binding oxygen molecules to each of its heme groups.[28]

However, when hemoglobin is deoxygenated, nitric oxide can bind to the heme groups, forming iron nitrosyl complexes. Together with the ability of NO to nitrosate the available cysteine thiol on the beta subunits to give S-nitrosohemoglobin (SNO-Hb), hemoglobin is able to scavenge NO in the blood.[29] Besides its ability to bind NO, however, the deoxygenated form of hemoglobin (deoxy-Hb) was also described to be able to act as a nitrite reductase.[30–32]

$$NO_2^- + Fe(II)Hb + H^+ \rightarrow NO + Fe(III)Hb + OH^- \quad \text{(I)}$$

This reaction yields equimolar concentrations of nitric oxide and methemoglobin (metHb), which has ferric iron as its prosthetic group and leads to the formation of R-state hemoglobin. This R-state hemoglobin is able to drive the reaction with nitrite faster than T-state hemoglobin, which compensates for the loss of ferrous hemoglobin in favor of metHb. [31,32]

In contrast to hemoglobin, the muscle pigment myoglobin contains only a single heme group that is able to bind an oxygen molecule. Myoglobin, a cytosolic protein rather than circulating in the bloodstream, is used for oxygen storage rather than transport. Despite these differences, myoglobin is able to bind nitric oxide to its single heme group in its ferrous, deoxygenated state with a high affinity.[33] Similarly, it can reduce nitrite to nitric oxide as deoxymyoglobin (deoxyMb), albeit at a speed that is up to 30 times faster than that of the reaction with deoxyhemoglobin.[34]

$$NO_2^- + Fe(II)Mb + H^+ \rightarrow NO + Fe(III)Mb + OH^- \quad \text{(II)}$$

Regeneration of the ferric metmyoglobin (metMb) to deoxyMb, which can then further reduce nitrite to NO, requires the presence of NADH.[35]

As a monoflavin protein, xanthine oxidoreductase (XOR) forms a homodimer consisting of a single FAD group connected to a molybdenum site via two Fe/S clusters. At the FAD site, both the oxidation of NADH to NAD^+ and the generation of superoxide ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) from oxygen take place.[36]

On the other hand, both the two last steps of the purine degradation, the oxidation of hypoxanthine to xanthine and a second oxidation of xanthine to uric acid, as well as the reduction of nitrite to NO take place at the molybdenum site, which consists of a single Mo atom that is coordinated with a sulfur atom, two oxygen atoms and a derivative of pterin with a cyclic dithiolene group. The two non-identical ferredoxin iron-sulfur clusters connect the Molybdenum site and the FAD group and facilitate electron transfer between them.[36]

XOR can be found in tissues, blood and vascular endothelial cells in one of two isoforms, either xanthine oxidase (XO) or xanthine dehydrogenase (XDH). While XOR is expressed from a single gene and occurs mainly as XDH in normal tissues, XDH can be reversibly switched to XO under conditions of low oxygen availability, such as ischemia.[37]

In the XDH form, the enzyme can readily use NAD^+ as an electron acceptor and reduce it to NADH; however, this affinity for NAD^+ is greatly reduced in the XO form. In the presence of oxygen, XO will instead take an oxygen molecule as the electron acceptor to give superoxide.[38]

While XOR was previously known in redox biology mostly for its ability to produce reactive oxygen species, e.g. superoxide, it has been found to be able to react as a nitrite reductase under certain conditions.[39,40]

$$NO_2^- + Mo(IV) + H^+ \rightarrow NO + Mo(VI) + OH^- \qquad \text{(III)}$$

This reduction of nitrite to nitric oxide is favored under severely hypoxic or anoxic conditions when either xanthine or NADH is present as a substrate for electron transfer. Together with the fact that the ideal pH for NO formation from by XOR at 7.0 for NADH substrates and at around 6.0 for xanthine substrates,[41] the XOR enzyme undergoes a “hypoxic switch” of favoring different substrates at low oxygen tension (Figure 4). At severely hypoxic or anoxic conditions and with increasing acidity and NADH concentrations, XOR switches from preferentially oxidizing xanthine to uric acid at the Mo site and the formation of superoxide and hydrogen peroxide at the FAD group to increasingly oxidizing NADH to NAD^+ at the FAD site in order to reduce nitrite to NO at the molybdenum site. Therefore, the conditions of decreasing pH and oxygen availability at the site of ischemia make for ideal circumstances for XOR nitrite reductase activity.[42]

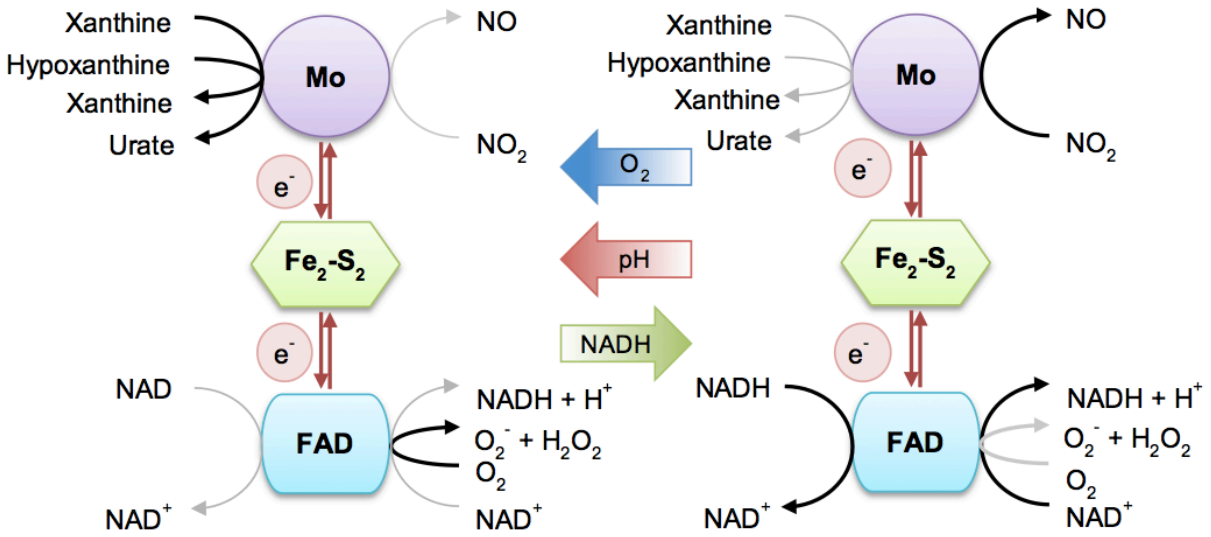


Figure 4 Hypoxic switch in xanthine oxidoreductase
At low oxygen tension, acidic pH and increased NADH concentration, xanthine oxidoreductase undergoes a "hypoxic switch" from favoring the conversion of hypoxanthine and xanthine at the Mo-site to free up electrons to be transported through the enzyme to the FAD group, where they are used to form superoxide, to preferring NADH as a substrate at the FAD site to reduce nitrite to NO at the Molybdenum group. Adapted from [42]

An important consideration in the biology of NO is its extreme instability and short-lived nature caused by its high reactivity as a free radical. It only exists for an average of 6 – 10 seconds before undergoing a depletion reaction. This depletion is due to scavenging of NO by various compounds or due to conversion to other nitrogen species such as nitrite (NO_2^-), nitrate (NO_3^-) and peroxynitrite ($ONOO^-$).[14]

Apart from the previously described scavenging of NO by binding hemoglobin and myoglobin, both hemeproteins can also oxidize NO in their oxygenated state (oxyHb, oxyMb) to give nitrate and metHb or metMb.[43]

$$NO + (Fe(II) - O_2)Hb + H^+ \rightarrow {NO_3}^- + Fe(III)Hb + OH^- \quad \text{(IV)}$$

$$NO + (Fe(II) - O_2)Mb + H^+ \rightarrow {NO_3}^- + Fe(III)Mb + OH^- \quad \text{(V)}$$

A pathway of oxidizing NO to another one of its storage forms, nitrite, is by reaction with hypervalent metal complexes, which are produced by oxidation of metal-oxygen species by oxidizing agents such as H_2O_2.[14]

NO depletion can also play a protective role in lipid peroxidation by reacting with the therein formed alkyloxy or peroxy radicals to give a stable product and thus terminating further peroxidation reactions.[44]

$$NO + LOO^{\cdot} \rightarrow LOONO \quad \text{(VI)}$$

In the presence of oxygen, NO can also oxidize to other potentially harmful nitrogen species such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3), the latter of which can then undergo hydrolysis in aqueous solution.[45,46]

$$2\,NO + O_2 \rightarrow 2NO_2 \quad \text{(VII)}$$

$$NO + NO_2 \rightarrow N_2O_3 \quad \text{(VIII)}$$

$$N_2O_3 + H_2O \rightarrow 2HNO_2 \quad \text{(IX)}$$

As with molecular oxygen, NO can also react with superoxide to form peroxynitrite, which can then further react with another NO or oxygen molecule to give nitrogen dioxide.

$$NO + O_2^- \rightarrow ONOO^- \quad \text{(X)}$$

$$ONOO^- + NO \rightarrow NO_2^- + NO_2 \quad \text{(XI)}$$

$$ONOO^- + O_2^- \rightarrow NO_2^- + NO_2 + O_2 \quad \text{(XII)}$$

1.3. Gasotransmitters

In recent years, small, endogenously generated gaseous molecules, named gasotransmitters, gained interest from researchers in various biomedical fields for their role in different cell signaling processes. In order to be classified as such, they have to be small gaseous molecules that are membrane permeable, meaning they do not require any transportation molecule or membrane receptors. Furthermore, they have to be endogenously produced at a regulated physiological level for specific signaling purposes and act on specific targets on a cellular or molecular level. Lastly, these actions have to be able to be reproduced by applying the gases from exogenous sources.[47–49]

Among these molecules, nitric oxide was the first to have been found to take up a function in signaling, followed by carbon monoxide (CO)[50] and hydrogen sulfide (H_2S)[51]. Apart from these better-researched signaling gases, other candidates for gases with biological functions such as methane (CH_4)[52] and ammonia (NH_3)[53] were found. Most of these gases were previously only known for their role in environmental chemistry, e.g. the greenhouse gases CO, NO, and CH_4, before discovering their pathways of endogenous production and their biological targets.

Like nitric oxide, which was already discussed at length in a previous chapter, carbon monoxide was discovered in the early days of gasotransmitter research. It was previously mostly known for its asphyxiating effect, which is due to CO reversibly binding to hemoglobin to form carboxyhemoglobin (CO-Hb), thereby blocking oxygen transport through the bloodstream;[54] however, it was found to be endogenously produced as a byproduct of hemoglobin degradation by the inducible heme oxygenase-1 (HO-1) and the constitutively expressed heme oxygenase-2 (HO-2). Another similarity to NO is the fact that CO has been found to partake in neural signaling and regulation of vascular tone and protect from ischemia/reperfusion injury.[55,56]

Another gasotransmitter to attract great research interest was hydrogen sulfide, a gas previously associated mostly with its pungent odor of rotten eggs, which is toxic at higher concentrations. It has been found to be produced in mammalian cells mainly by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), using L-cysteine as a substrate.[57] These enzymes are differentially expressed in various tissues, such as vascular tissue, intestine, brain, kidney, and liver.[57,58] Like NO and CO, H_2S can be scavenged by hemoglobin, binding to metHb to give sulfhemoglobin.[59] Furthermore, it has also been shown to act as a vasodilator and to play a part in the regulation of blood pressure,

especially under hypoxic conditions, as well as to have a function in neurotransmission and neuromodulation.[51,60]

One of the most recent discoveries in the field of gasotransmitters is ammonia. Well-known for its strong, stinging smell, it is produced at physiological concentrations by a variety of deamination enzymes in different tissues. In neurons, for example, ammonia is produced from phosphate-activated glutaminase using glutamine as a substrate, while glutamate dehydrogenase is responsible for forming NH_3 in astrocytes.[61] It can be removed from the system by direct excretion from urine. Besides its functions as a regulator of redox and pH balance and source of nitrogen for DNA and RNA syntheses, a signaling function as an inducer of posttranslational modifications of proteins in astrocytes has been observed. [53,62]

As a final example of a gasotransmitter, methane was discovered to have biological functions only in recent years. Like many other described gasotransmitters, the abundant organic gas is well known for its role as a greenhouse gas and as a compound in natural gas. It, among others such as carbon dioxide and diatomic hydrogen gas, is also one of the most abundant gases present in the intestines.

The endogenous source of CH_4 is methanogens, such as *Methanobrevibacter smithii* via a reaction of hydrogen gas with carbon dioxide, and other gut bacteria, which are present in about one-third of adults.[63,64] Furthermore, it has been shown to be produced not only in plants and eukaryotic cells but also in mammalian cells.[65]

CH_4 has been implicated as a factor in a number of gastrointestinal conditions, e.g. colorectal cancer and inflammatory and irritable bowel disease, where it has been shown to slow down transit through the bowels and influence the contractions of the muscles in the intestine.[66,67] Other studies have linked methane to anti-inflammatory and cytoprotective effects in an ischemia/reperfusion model, where neutrophil infiltration and production of ROS, such as superoxide, in oxidative stress were found to be reduced.[52,68]

A possible mechanism of an interplay between the gasotransmitters NO, H_2S and CO has been proposed, putting forward the idea that some, if not all, gasotransmitters are interconnected.[69,70]

Due to their favorable effects in reducing cell and tissue damage, a number of gasotransmitters are being researched as prospective therapeutic gases in ischemia/reperfusion injury (Figure 5).

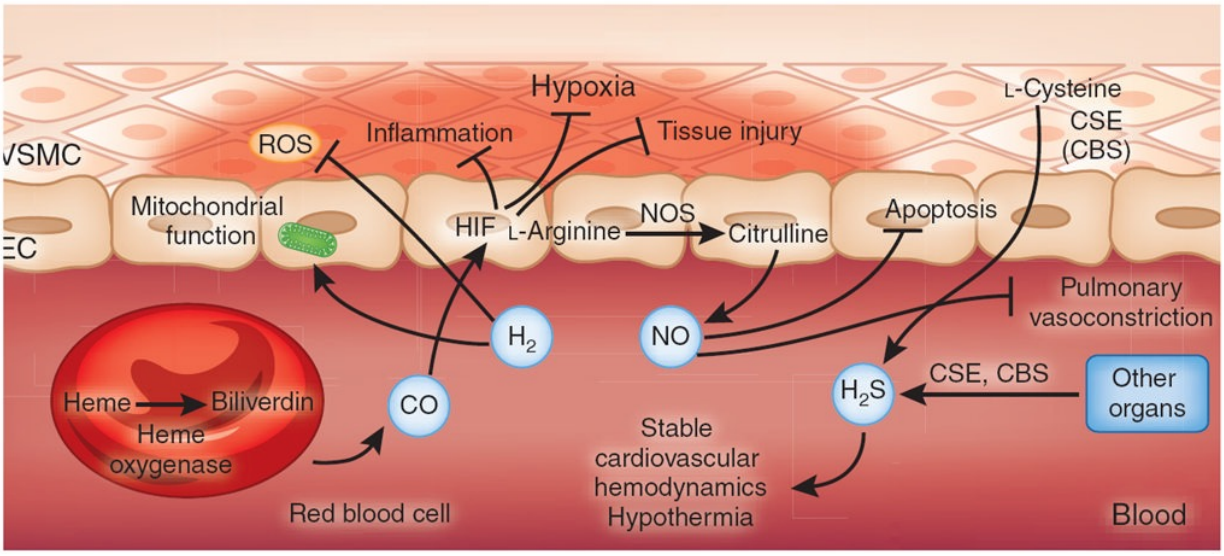


Figure 5 Possible applications of gasotransmitters as therapeutic gases
While nitric oxide therapy by inhalation of NO gas is used to decrease apoptosis of cells and dilate vessels to prevent blocking the circulation, H_2S has been used for its cytoprotective effect and to aid stabilization of blood flow in the cells. Carbon monoxide has been used to positively influence stabilization of the Hypoxia-Inducible Factor (HIF), which is an important mediator of responses of cells to hypoxia, as well as to reduce inflammation. Additionally, hydrogen gas, while technically not meeting all criteria to be considered a gasotransmitter as no endogenous target for it has been identified, is used to decrease ROS formation and preserve physiological mitochondrial function. Adapted from [71]

1.4. NO detection methods

Due to its role in many physiological pathways and excess production in many pathological defense mechanisms, e.g. in shock and inflammation, detection and quantification of nitric oxide can yield important information about ongoing processes in cells and tissue. For this purpose, various detection methods and techniques for different diagnostic purposes have been developed over the years.

2.2.1. Griess reaction

The colorimetric Griess reaction is among the oldest and most widely known detection techniques for nitric oxide species. It employs the previously described auto-oxidation of NO to nitrite in the presence of oxygen and acts based on the assumption that NO_2^- is a stable oxidation product. The detection of nitrite is a two-step process, which first involves the formation of a nitrosating agent from nitrite under acidic reactions, which reacts with sulfanilamide to give a diazonium salt. This salt can then undergo a coupling reaction with the aromatic amine N-(1-naphthyl)ethylenediamine dihydrochloride, resulting in a colorimetrically detectable azo dye.[72]

2.2.2. Fluorescence-based detection

As a more sensitive NO detection method, a number of fluorescent dyes have been developed that are based on the ability of NO to N-nitrosate compounds. Of these agents, 2,3-diaminonaphthalene (DAN) and the family of diaminofluoresceines (DAF) have gained the most favor from researchers. The 2,3-naphthotriazole formed during the reaction of DAN with NO yields a strong fluorescent signal about 100-fold higher than that of the unreacted DAN dye when excited at 375 nm and the emission monitored at 415 nm. [73]

In contrast, the different DAF compounds do not exhibit a big shift in the absorbance maximum when reacting with NO, but rely solely on the great increase in fluorescent signal. Their advantage over DAN dye is the use of excitation wavelengths in the visible spectrum, which proves to be less harmful to the cells and removes any chance of interference of biological auto-fluorescence.[74]

Both dye forms are highly specific to NO, as other nitrogen species such as NO_2^- and NO_3^- do not induce the shift in fluorescence seen in the reaction with NO. Together with their high sensitivity, the use of fluorescent dyes for NO detection makes for a very versatile method.[73,74]

2.2.3. Electron paramagnetic resonance

Electron paramagnetic resonance (EPR), also known under the names electron spin resonance (ESR) and electron magnetic resonance (EMR), is a method for the detection of free radicals and utilizes the shift in the absorption spectra of electromagnetic radiation specific to the bonds of radicals with a paramagnetic compound, called a "spin trap". There are a great number of different spin traps for various molecules, including iron and heme complexes for NO detection.[75] The most common trap for NO is iron complexes of diethyldithiocarbamate. As it is an insoluble complex in aqueous solutions, it is introduced as two components, an iron salt which is soluble in water and diethyldithiocarbamate. There is also a water-soluble analog of this trap, N-methyl-D-glucamine dithiocarbamate. The oldest trap for NO is hemoglobin. NO can be tightly bound to deoxygenated ferrous hemoglobin to form HbNO complex, which has distinct EPR-detectable signal.[76]

2.2.4. Chemiluminescence-based detection

A highly sensitive system for both continuous monitoring of NO formation and measurements of the contents of nitric oxide species in individual samples is the chemiluminescence-based detection of nitric oxide. One route to induce chemiluminescence is the use of a hydrogen peroxide/luminol system, in which H_2O_2 first oxidizes NO to peroxynitrite, which then undergoes a reaction with luminol that induces chemiluminescence. With a detection limit of about 100 fM, this is a very useful method for detection of nitric oxide in a low concentration range.[77]

Another chemiluminescence-based detection system is based on the reaction of ozone with NO. In this method, nitric oxide reacts with ozone generated from air to result in nitrogen dioxide, which is in an excited, high-energy state and oxygen.

$$NO + O_3 \rightarrow NO_2^* + O_2 \qquad \text{(XIII)}$$

This excited nitrogen dioxide is very unstable due to its excess energy and decays to its ground state, thereby emitting a single photon.

$$NO_2^* \rightarrow NO_2^* + h_\nu \qquad \text{(XIV)}$$

This photon is then directed to a photomultiplier tube and recorded as a signal directly proportional to the amount of NO in the sample.[78] The specificity and reliability of this method have promoted the technique to one of the most widely used to this date, both for diagnostic and research purposes.

1.5. Aims of this study

The general aim of this study was to clarify the mechanisms underlying the generation of NO in tissues under hypoxic conditions.

The general aim was broken into three parts:

1. Characterization of NO release from nitrite under hypoxic condition in different tissue homogenates.
2. Identification of the major source of NO in the ischemic intestine.
3. Investigation of a possible effect of the presence of methane on NO release in intestine homogenate.

Upon its completion, this project shall shed light on the mechanisms of ischemic NO production and show a possible interaction between the two gasotransmitters nitric oxide and methane.

2. Materials and Methods

2.1. Sampling of tissues and preparation

All tissues and blood used in the course of the following experiments were collected from male Sprague Dawley rats (300 – 550 g; Charles River, USA) in accordance with the Guide for the Care and Use of Laboratory Animals set by the National Institutes of Health. The rats were kept under standard housing conditions which continual access to standard rodent food and drinking water. Upon arrival from the company to the laboratory animal care facility, they were kept for a minimum of one full week before use in experiments in order let them acclimatize to the new surroundings and to rule out any transport-related stress.

2.2.1. Anesthesia

Prior to anesthesia, the rats were weighed and carefully sat into a plastic container draped over with a cloth to minimize stress for the animal. The anesthesia was then induced by connecting the box to a tube causing an influx of about 800 mL/min of a gas mixture containing 1 – 3.5 % isofluorane (Forane, AbbVie GmbH, USA) in oxygen-enriched air. After 5 to 10 minutes of ventilation, depending on the size of the animal, the depth of anesthesia was confirmed first by observing the breathing patterns, then gently moving the animal around in the closed box and looking for any reactive movement. Only then was the box opened, the corneal reflex checked to confirm sufficient sedation.

2.2.2. Blood and tissue sampling

Euthanasia was executed by decapitation of the deeply anesthetized animals on a guillotine for small rodents. The fresh blood was collected from the bodies of the rats into a 50 mL plastic Falcon tube (Corning Inc., New York, USA) containing 200 µL Heparin (1000 U/mL, Gilvasan Pharma, Austria), thoroughly mixed by repeated careful inversion of the tube and stored on ice for the measurements.

For the collection of perfused liver samples, the rats were anesthetized and euthanized as described above. Following the dissection of the animal, the *Vena hepatica* was cannulated (Venflon 20 G, BD Europe, Switzerland) and perfused by hand using plastic syringes containing Ringer solution (Fresenius Kabi, Germany) containing 0.8 U/mL Heparin until the blood was visibly drained from the tissue and the liver took on a uniformly pale color, all the while taking care not to apply excess pressure to avoid rupturing the vessels and tissue. The tissue was then excised and placed into a beaker containing ringer solution. Pieces of liver were then cut up into pieces of 1 – 2 mm^2 on a clean plastic petri dish placed on top of an ice bed, immediately frozen in liquid nitrogen and stored at -80 °C in 2 mL Cryo.s tubes (Greiner bio-one, Austria).

The non-perfused liver samples were obtained as described above; however, the liver was excised immediately after decapitation without the perfusion step. It was removed from the body, rinsed in a beaker of Ringer solution and cut up and stored at -80 °C.

For the measurements on small intestine samples, the small intestine from *duodenum* to *ileum* was resected from the rat intestines and placed in Ringer solution. Using plastic syringes containing fresh Ringer solution, the fecal matter and contents of the intestine were flushed out thoroughly by repeated rinsing. Only then was the tissue placed onto an ice-cold petri dish and cut up into small sections of 1 – 2 mm^2. The samples were then immediately frozen in liquid nitrogen, collected into Cryo.s tubes and stored at -80 °C for future use.

The heart tissue was collected by excising the fresh hearts from the bodies of the rats and quickly rinsing them in Ringer solution. On a clean, iced petri dish, the *auriculae atrii* were then removed with a scalpel and the remaining blood in the *ventricles* and *atria* was flushed out using a plastic syringe containing Ringer solution. The thus cleaned tissue was then cut up, frozen and stored as described above.

2.2.3. Sample preparation

The homogenate used in the liver and heart measurements was prepared by weighing the frozen tissue samples on a precision scale and adding 4 times the amount of an incubation buffer (80 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-HCl, 1 mM Diethylenetriamine pentaacetic acid; pH 6.0 or 7.4) to obtain a 1:5 dilution in the homogenate, all the while taking care not to thaw the samples by keeping them on ice whenever possible. Using a clean glass Potter homogenizer, the homogenization was then carried out by slowly moving the glass vessel 10 times up and down the homogenization rod without taking it completely out of the sample, so as not to create a vacuum in the liquid and thus avoiding rupture of any remaining red blood cells in the tissue samples.

As intestine proved to be too tough to be properly homogenized by a Potter homogenizer, the homogenization for these samples was carried out by pulverizing the tissue in a stone mortar and pestle under the repeated addition of liquid nitrogen to keep the samples frozen. The thus obtained intestine homogenate was then weighed and diluted by adding 4 times the amount of the incubation buffer described above. Only then was the Potter homogenizer used to properly mix the homogenized tissue with the buffer.

Homogenization was carried out in bulk and the obtained homogenates were aliquoted for use in the measurements and kept frozen at -80 °C or in liquid nitrogen until they were used in the measurements.

2.2. Measurements on the Nitric Oxide Analyzer

2.2.1. Tissue measurements

Homogenates of the liver, heart, and small intestine samples were prepared as described above. For measurements on both heart and liver samples, an aliquot of 2.5 mL of the tissue homogenate and 37.5 µL of Antifoam C solution (Sigma Aldrich, Austria) were pipetted into a glass measurement chamber set up to allow for continuous "on-line" detection of NO release that was thoroughly scrubbed and rinsed with distilled water. The sample was then filled up to a total volume of 18 mL, taking into account the volume of $NaNO_2$ stock solution (100 mM) added during the measurement.

In order to block the NO scavenging of thiol groups in samples, the homogenate treated with different concentrations of either a N-Ethylmaleimide (NEM)/ethylendiamide tetraacetic acid (EDTA) mixture or a saturated solution of NO in bi-distilled water (Fresenius Kabi, Germany) prior to the start of the measurements.

For the inhibitor measurements, the reaction mixture was incubated with 100 µM allopurinol (Sigma Aldrich, Austria), an inhibitor of xanthine oxidoreductase, or 30 µM myxothiazol (Sigma Aldrich, Austria), an inhibitor of cytochrome bc1, and equilibrated under the carrier gas (N_2 or N_2 containing 2.2 % CH_4) for 10 minutes prior to the start of the measurement.

For the start of the measurement, exact amounts of a 100 mM $NaNO_2$ stock solution were added through a septum in the reaction chamber and the amount of NO purged from the sample continuously measured by a chemiluminescence reaction in the Nitric Oxide Analyzer (Sievers 180i) for 15 to 30 minutes.

2.2.2. Myoglobin measurements

Prior to using the commercially bought myoglobin from horse heart (Sigma Aldrich Austria) to convert nitrite to NO, the myoglobin had to be reduced to deoxy-Mb by addition of sodium dithionite and removal of thus resulting side products using a dialysis cassette (MW 7.000, Thermo Scientific) in a solution of incubation buffer under 4 °C which was changed every two hours and let sit overnight.

The deoxy-myoglobin collected from the solution was used at a physiologically relevant concentration of 35 µg/g tissue for the measurements. Buffer and Antifoam C solution were added as described above, incubated with 100 mM allopurinol for xanthine oxidase inhibition and equilibrated under the carrier gas for 10 minutes.

The measurement was started by the addition of 15 mM $NaNO_2$ stock solution to the sample in the closed system and the resulting NO monitored for 15 minutes.

2.2.3. Xanthine oxidoreductase measurements

For the enzyme measurements, a final concentration of 1 or 4.4 mM $NaNO_2$ was added to 16 mL of incubation buffer (pH 6.0) and equilibrated for 10 minutes. After 9 minutes of equilibration, either xanthine (5, 10 or 20 µM; Sigma Aldrich, Austria) or NADH (0.2 or 1 µM; Sigma Aldrich, Austria) was added and the reaction was started by addition of 2 µg/mL xanthine oxidase (Sigma Aldrich, Austria).

In the measurements with the additional electron acceptor NAD^+, 10 µM was added at 8 minutes, 20 µM of NAD^+ at 9 minutes and the reaction was started by addition of 2 µg/mL xanthine oxidase.

The resulting NO under N_2 and N_2/CH_4 atmosphere was monitored for 15 or 10 minutes, respectively.

2.2.4. Simulation of physiological substrate ratios

In order to try and emulate the physiological ratios of substrates (xanthine, hypoxanthine, NADH and NAD^+) present in the tissue, the intestine homogenate was denatured and thereby rendered enzymatically inactive by boiling it in a water bath for 5 minutes.

The resulting denatured homogenate was then placed in the reaction chamber containing 37.5 µL Antifoam C and 16 mL incubation buffer (pH 6.0) and equilibrated for 10 minutes. After equilibration, 1 mM $NaNO_2$ was added and the slight rise in signal measured until stable before addition of 10 µM xanthine in order to account for any substrate destroyed in the denaturation process and 2 µg/mL xanthine oxidase at 8 minutes into the measurement.

The NO purged from the sample by both pure nitrogen and N_2 containing methane as a carrier gas was measured for a total of 15 minutes.

To achieve inhibition at the FAD site, the denatured homogenate was incubated with 100 µM diphenyleneiodonium chloride (DPI; Sigma Aldrich, Austria) and the measurement conducted as described above.

2.2.5. NO Donors

To assess a possible change in NO scavenging in the tissue samples upon the addition of CH_4 to the carrier gas, a sample mixture containing 250 µL small intestine homogenate and 100 µM allopurinol and brought to a total volume of 16 mL with incubation buffer (pH 6.0).

The release of NO from 25 µL of a 4.4 mM DETA-NONOate stock solution was measured under both N_2 and N_2/CH_4 atmosphere for 15 minutes.

3. Results

3.1. Release of NO in different tissues

Figure 6 shows a graph taken from the paper “Impact of mitochondrial nitrite reductase on hemodynamics and myocardial contractility”, which was co-authored by the author of this thesis. For this study, she determined the kinetics of NO release from nitrite in different tissues. These results are published in the journal Scientific Reports. An interesting phenomenon, which was observed, but not explained in this paper, was a delay, termed the lag phase, in the release of NO after addition of nitrite from tissue to tissue and was increased with increasing oxygen levels. In the following experiments, the author has addressed this question after establishing a corresponding method.

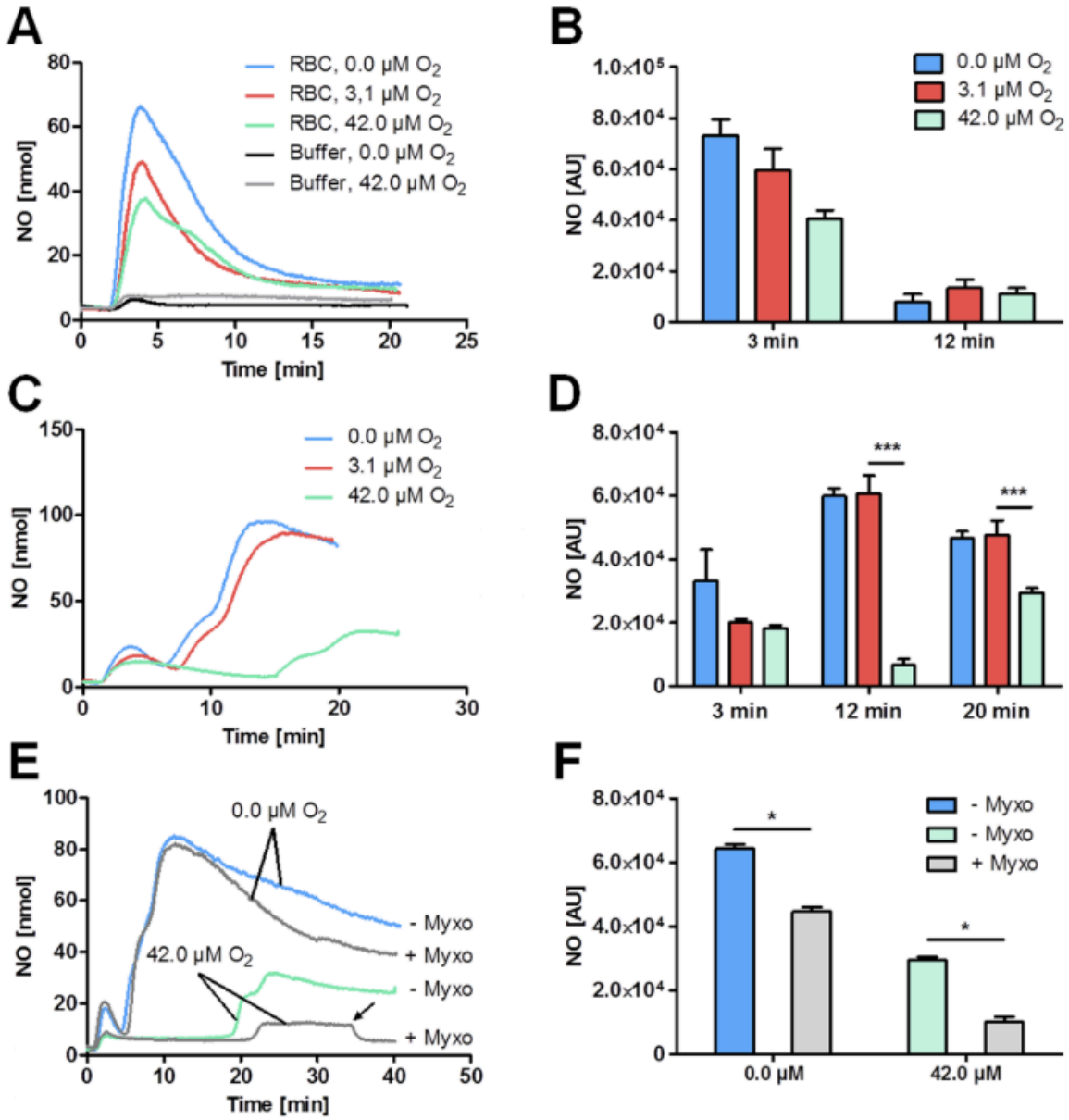


Figure 6 Effect of different oxygen concentrations on NO release from nitrite reduction in erythrocytes and heart homogenates[79]

(A,B) NO release and quantification in erythrocytes at oxygen concentrations of 0.0 µM (blue line), 3.1 µM (red line) and 42.0 µM (green line). The black and grey lines depict the NO signal from nitrite in incubation buffer. (C,D) NO release and statistical analysis from heart homogenates at the different oxygen concentrations.

(E,F) Contribution of mitochondria to NO release in heart homogenates was assessed by addition of the mitochondrial complex III inhibitor myxothiazol (myxo, grey lines) at oxygen concentrations of 0.0 µM and 42 µM. The arrow in (E) marks the addition of the complex IV inhibitor potassium cyanide.
Data are expressed as mean ± SEM of at least n = 5, *p < 0.05, **p < 0.01, ***p < 0.001.

3.2. Establishing the method

In an effort to characterize the origin of NO and kinetics of NO production, the Nitric Oxide Analyzer (NOA, Sievers 280i, General Electrics Instruments, USA) was used to measure the nitric oxide continuously purged from non-perfused liver homogenates upon stimulation of NO production using 2.1, 4.4, 8.6 and 15.6 mM sodium nitrite.

A characteristic delay between the addition of sodium nitrite and the beginning of the bulk release of NO from the sample, henceforth called the lag phase, was gradually decreasing if the concentration of nitrite added was increased (Figure 7). With higher concentrations of nitrite, the lag phase was shortened significantly from about 14 min at 4 mM $NaNO_2$ to about 4 min at 15.6 mM $NaNO_2$.

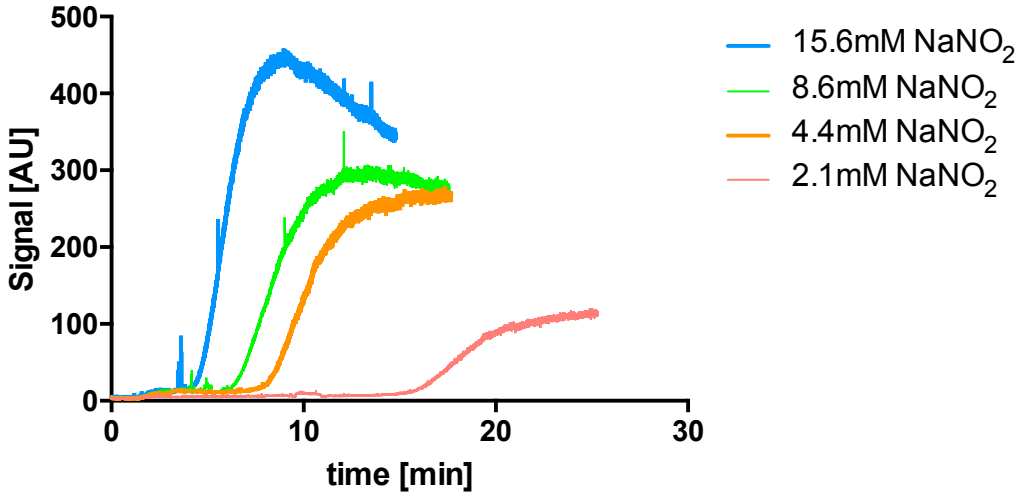


Figure 7 Effect of nitrite concentration on NO release
In liver homogenates, an increasing amount of $NaNO_2$ used to stimulate NO production led to increases in the slope and overall NO produced as well as a decrease in the lag phase, a period of time between addition of sodium nitrite and the beginning of NO release. An approximately 7.5-fold increase of nitrite from 2.1 mM to 15.6 mM led to a shortening of the lag phase from 14 minutes down to 4 minutes.

We assumed that the delay in the NO release is due to initial binding NO to the targets in homogenate. The reaction between thiol (-SH) groups and NO to form –SNO complexes is the most common reaction of NO occurring in biological systems. To clarify whether or not this reaction is responsible for lag phase we incubated homogenates with a mixture of N-ethylmaleimide (NEM) and ethylenediaminetetraacetic acid (EDTA) for 10 min prior to the measurements in order to block thiol groups and prevent the formation of –SNO complexes.

Indeed the addition of increasing concentrations of NEM/EDTA shortened the lag phase, supporting our assumption (Figure 8). In addition, NEM/EDTA increased the overall amount of NO released from the sample, suggesting that –SH groups are a key scavenger of NO in homogenates tested. However, a control experiment (**Supplementary Figure 1**) showed a strong NO signal just from adding NEM to incubation buffer, even without the presence of

nitrite or homogenate. Therefore NO scavenging was investigated further in the following experiment.

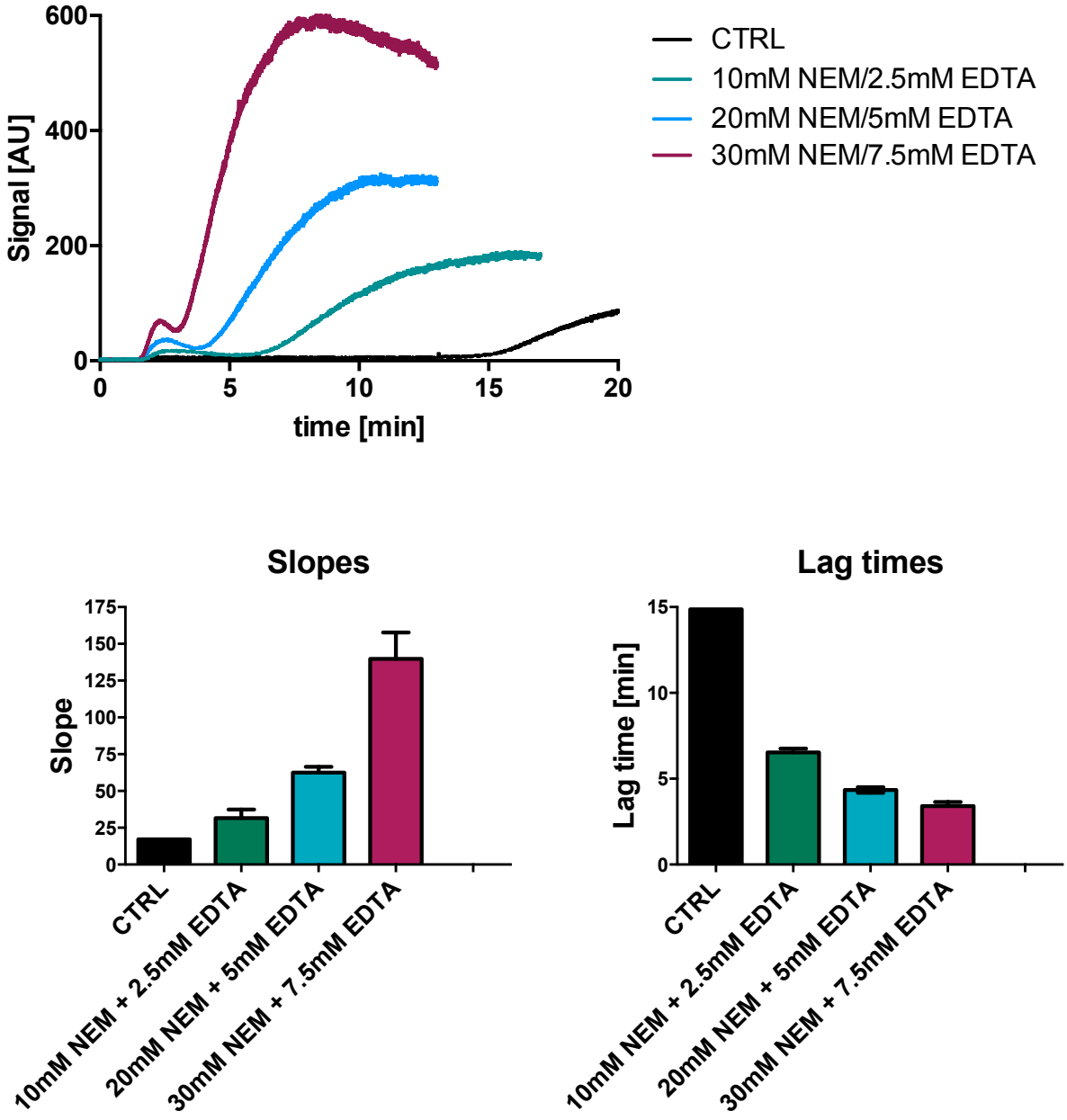


Figure 8 Effect of thiol-blocking solution on NO release
With increasing concentrations of a NEM/EDTA thiol group blocking solution, the lag phase was significantly shortened from 14 min in the control down to nearly 0 min at a concentration of 10 mM NEM/2.5 mM EDTA. However, despite using the same concentration of nitrite (2.1 mM) in all measurements the magnitude of the NO signal increased with increasing concentrations of NEM/EDTA.

In another attempt to pinpoint the existence of the lag phase to scavenging in the tissue, the homogenate samples were pre-incubated with 100, 200 and 300 µL of a saturated (2 mM) NO solution in order to pre-saturate the thiol groups (and possibly other groups) able to bind NO prior to the measurement of the reduction of nitrite to NO. This treatment completely abolished the lag phase, suggesting that the lag phase in NO production from samples is indeed caused by scavenging of initially produced NO (Figure 9**)**. In contrast to experiments with NEM/EDTA, the slope did not change remarkably with increasing NO concentrations.

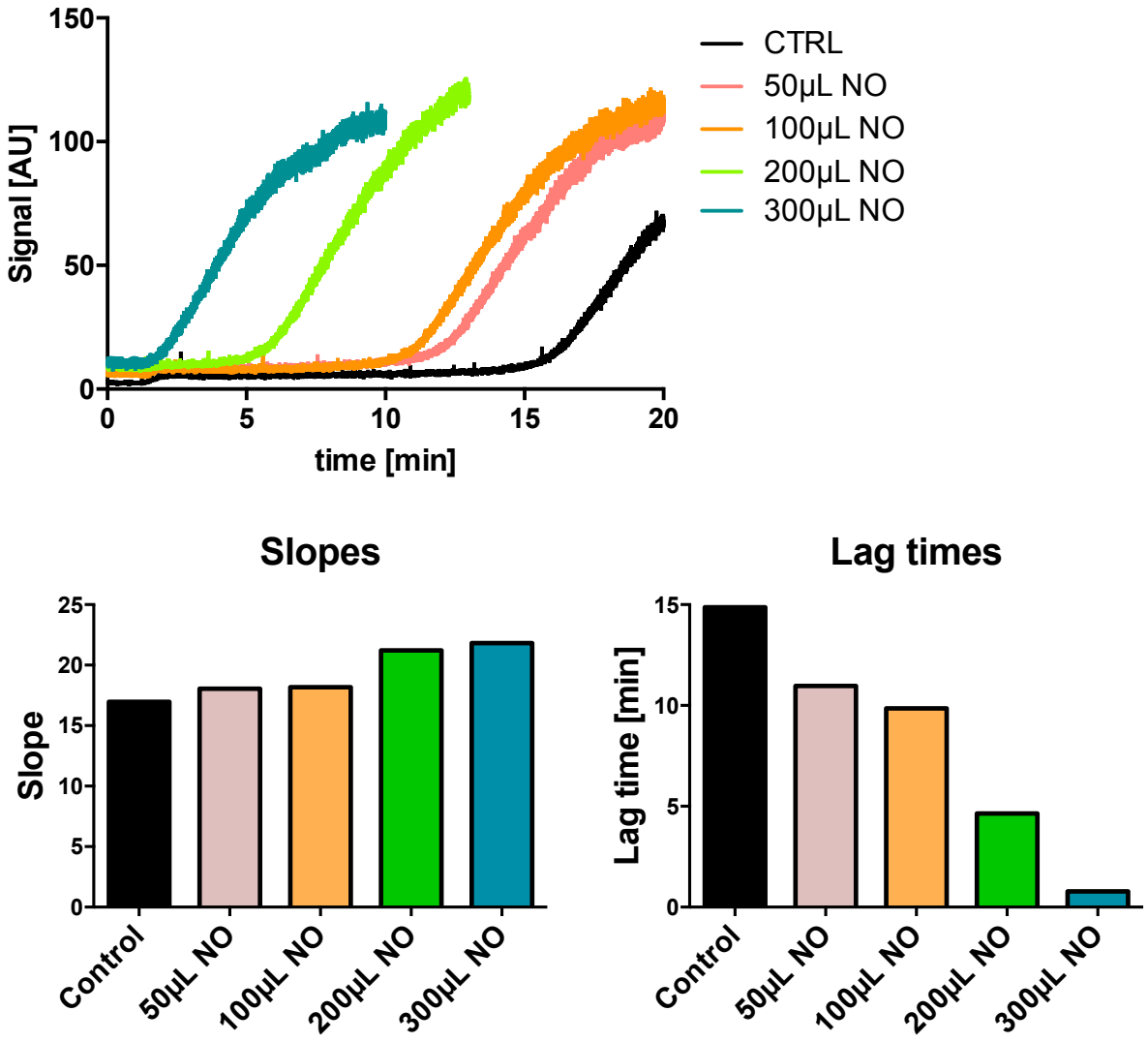


Figure 9 NO release from liver homogenate treated with NO solution
The NO release from liver homogenates that were incubated with different amounts of a saturated NO solution prior to the measurements, while overall maintaining similar slopes and magnitudes, showed significant dose-dependent reduction of the lag phase between the addition of 2.1 mM $NaNO_2$ and the bulk NO release from the samples, with lag times between 14 min and immediate release (0 min).

Another important scavenger of NO, hemoglobin, is in the blood. This suggests that the residual blood in the tissue may substantially influence the measurements of nitrite reductase activity. To estimate the effect of residual blood in the tissue on nitrite reductase activity measurements we compared perfused and non-perfused liver samples. The rat liver was perfused prior to excision using a Ringer solution with 0.8 U/mL heparin to reduce the amount of residual blood and thereby hemoglobin in the tissue samples and compared these measurements with the non-perfused liver. The results are displayed in **Figure 10**. We observed that in the perfused liver, the lag phase was drastically reduced, suggesting that most of the initial binding of NO is due to NO-Hb binding.

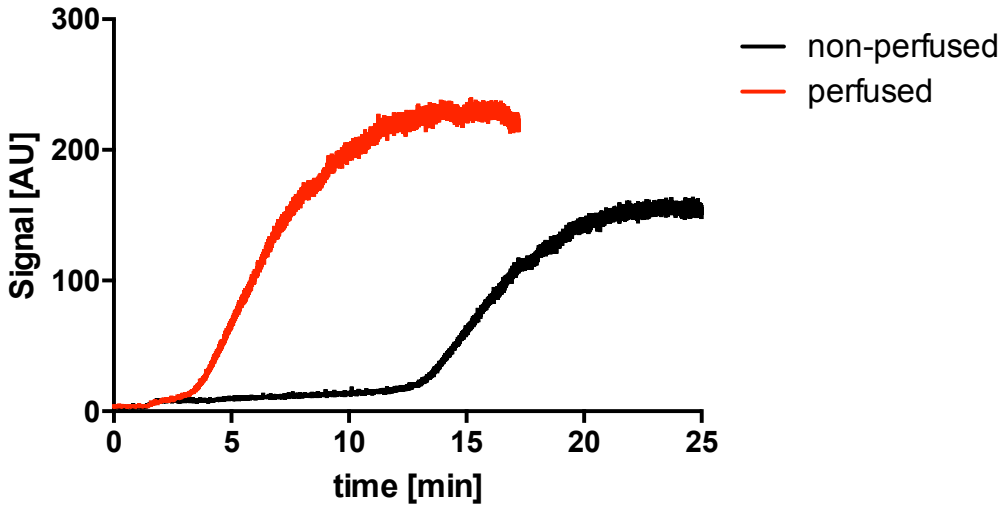


Figure 10 Comparison of NO release from perfused and non-perfused liver
While the NO release in non-perfused (blood containing) liver follows the typical shape of a significant lag phase of 10 – 15 minutes between the addition of nitrite and bulk NO release, this lag phase was shortened to 2 min in homogenate from perfused liver tissue.

This is in line with our observations, that lag phase does not occur in experiments with intestine homogenates, which retains much lower amounts of blood in the tissue. Another specific characteristic of the intestine is that it has extremely high nitrite reductase activity. The rate of NO release from the intestine is much higher than in the liver or heart homogenates (Figure 11).

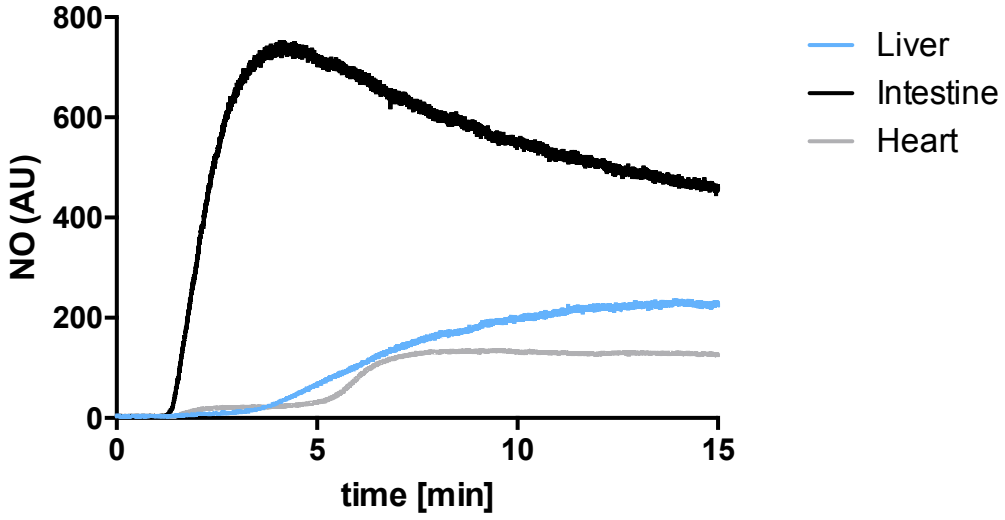


Figure 11 Comparison of NO release from heart, liver, and intestine
The NO release from homogenates containing liver at a dilution of 1:36 from 4.4 mM $NaNO_2$ is comparable in height with NO production from a 1:36 dilution of heart tissue stimulated with a more than 3 times higher concentration of 15 mM nitrite solution. NO release from small intestine was much higher, allowing for a total tissue dilution of 1:360 and the use of 4.4 mM $NaNO_2$.

In order to further optimize the measurements and aiming for better physiological comparability, the temperature of the reaction mixture was raised from room temperature at 22 °C to 37 °C by placing the glass reaction chamber in a water bath, which led to an increase in NO-release, meaning an optimized nitrite reductase activity at a physiologically relevant temperature.

Similarly, lowering the pH of the buffer from pH 7.4 to pH 6.0, which is more relevant under local ischemic conditions, was seen to significantly increase NO production in the tissue samples.

The thus optimized measurement conditions (37 °C, pH 6.0) were applied in all subsequent measurements unless stated otherwise.

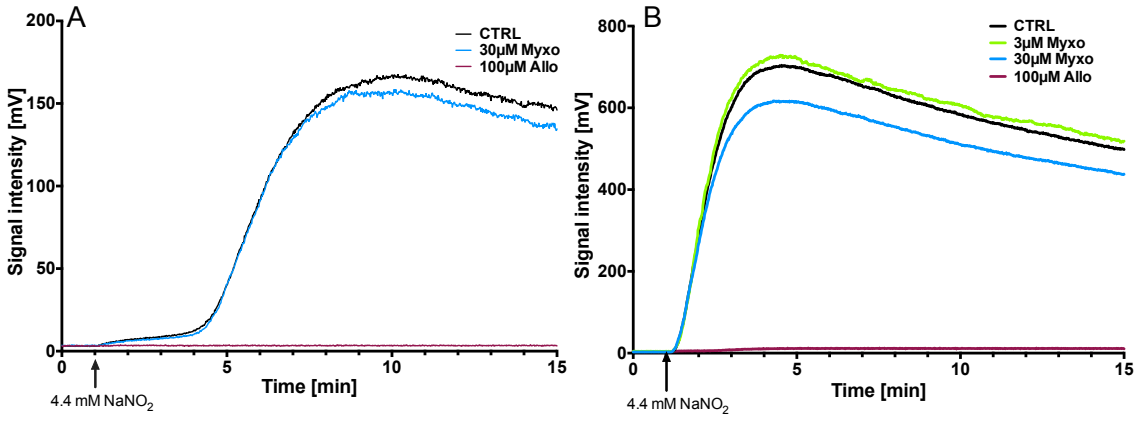
3.3. Investigating the source of NO

After establishing the method and investigating the kinetics of the NO detection as described above, the source of NO in different tissues was to be determined. For this purpose, the homogenate was incubated with either allopurinol, an inhibitor of XOR, or myxothiazol, an inhibitor of mitochondrial complex III, in order to eliminate any contribution of XOR or mitochondria to the NO released from the tissue.

In homogenate of the perfused liver, inhibition of the mitochondrial respiratory chain showed to have only a small effect on the overall NO produced. This was similar to what we published previously.[79] In contrast, inhibition of XOR led to near-complete inhibition of NO production (Figure 12**A**).

Similarly, while producing a much greater overall NO signal, calling for adjustments to the amount of tissue (250 µL or 10 % of the amount used for liver measurements) and nitrite (4 µM) used in measurements, the inhibition of cytochrome bc1 had little effect on NO production when using 3 µM myxothiazol and only exhibited greater inhibition when increasing the amount of inhibitor 10-fold. Allopurinol, on the other hand, inhibited the NO signal almost completely, suggesting that either myxothiazol or allopurinol exhibit off-target effects at higher concentrations (Figure 12**B**).

In measurements using heart homogenates, a much greater concentration of nitrite (15 µM) had to be used to compensate for the smaller NO production in the tissue. Similar to the liver homogenates, the contribution XOR to NO production was greater than the contribution of mitochondria. (Figure 12**C**).



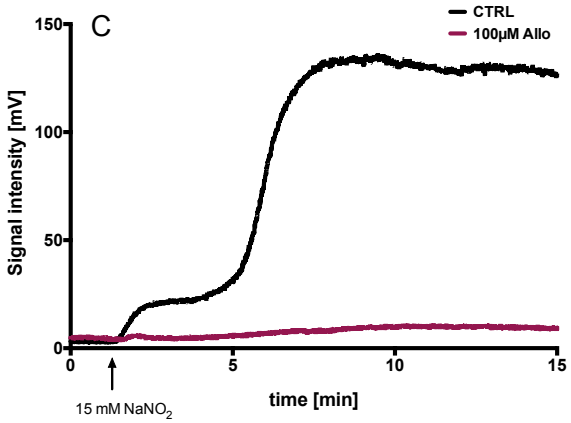


Figure 12 Effect of inhibition of NR on the overall nitrite reduction
(A) In perfused liver homogenates, the inhibition of mitochondrial cytochrome bc1 by 30 µM myxothiazol only prevented a small part of the overall NO formation. Incubation with the xanthine oxidoreductase inhibitor allopurinol, however, kept NO release from the sample near baseline levels.
(B) The inhibition of NO release by myxothiazol was not observed when using a concentration of 3 µM myxothiazol. However, this inhibition was significantly stronger by increasing the inhibitor concentration 10-fold to 30 µM. Incubation of homogenate with allopurinol led to a near-complete inhibition of NO signal at a concentration of 100 µM.
(C) The overall much smaller NO release, even when supplying three times as much nitrite (15 mM) as a substrate for reduction, was almost completely inhibited by addition of allopurinol.

Thus concluding that XOR seems to be the main contributor of XOR in liver, heart, and intestine tissue under ischemic conditions, the NO production capabilities of proposed nitrite reductases were investigated in an isolated environment.

As hemoglobin was seen to have a NO scavenging rather than producing effect in line with the scavenging in non-perfused liver tissue, the contribution of Hb to NO production was found to be insignificant.

In deoxygenated myoglobin (deoxy-Mb) from horse heart, the NO production signal was found to be very low at physiological concentrations of 35 µg/g myoglobin, even when using high concentrations of nitrite to stimulate NO production. This was similar to the low NO signal measured from heart homogenate samples. Upon addition of allopurinol, however, the NO production was almost completely inhibited, which suggests off-target effects of allopurinol on myoglobin (Figure 13).

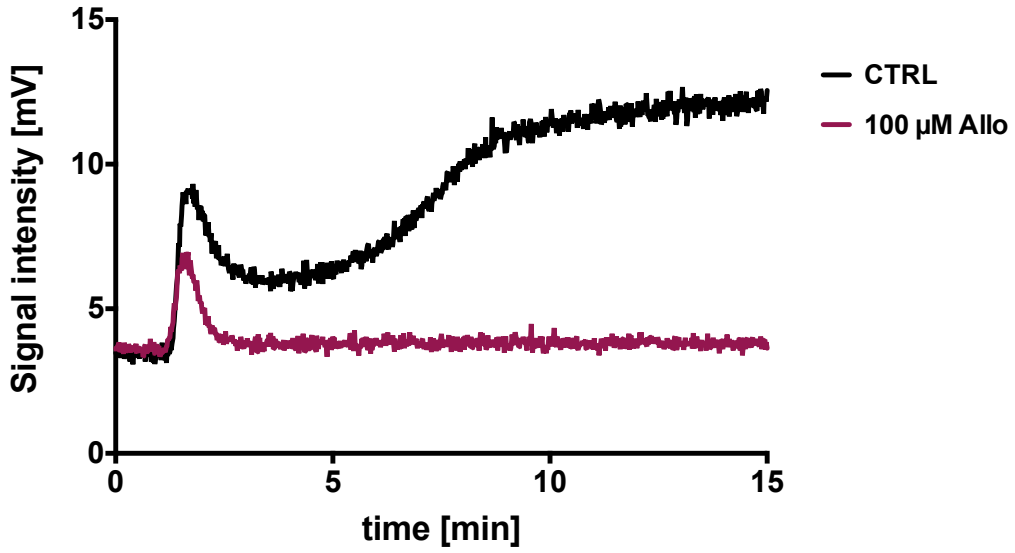


Figure 13 NO formation by isolated myoglobin
The NO release from isolated myoglobin from horse heart barely rises above baseline levels, even upon the addition of high concentrations of nitrite (15 mM). This NO release is completely inhibited in samples treated with 100 µM allopurinol, save for a small spike which represents the addition of nitrite.

In a pure enzyme system of XOR, several substrates are eligible as substrates for nitrite reduction. Both hypoxanthine and xanthine are oxidized at the Molybdenum site; however, hypoxanthine can undergo oxidation twice. The general appearance and magnitude of the rate of NO release by XOR using xanthine as an electron acceptor and nitrite as an electron donor was found to be very similar to the NO production in intestine homogenate, supporting the key role of XOR in hypoxic NO release.

The data presented above suggest XOR, rather than myoglobin and cytochrome bc1 contribute to the release of NO in this experimental model. As expected, this nitrite reduction of XOR is completely inhibited upon addition of XOR-inhibitor allopurinol.

3.4. Effect of methane on NO release

Recently, our laboratory has reported that intestinal ischemia causes an increase in NO levels in the intestine. Interestingly, the treatment of these animals with methane (CH_4) reduced the levels of NO.[80] Considering our data described above we hypothesize that the decrease in NO levels by CH_4 is due to inhibition of XOR. Thus, the next goal of this study was to investigate a suspected interaction between the metabolisms of the gasotransmitters NO and CH_4 and involvement of XOR in this process.

To measure the effect of methane on the NO production, the inert carrier gas N_2 (nitrogen) constantly bubbling through the homogenate in the reaction chamber of the NOA was exchanged for a gas mixture of N_2 containing 2.2 % CH_4. In a first experiment, the measurements of NO release from a XOR – xanthine enzyme system were repeated using the carrier gas mixture and xanthine concentrations of 5, 10 and 20 µM. As shown in Figure

14, the presence of CH_4 slightly increased the production of NO in the enzyme system. However, this effect decreased with increasing substrate concentrations.

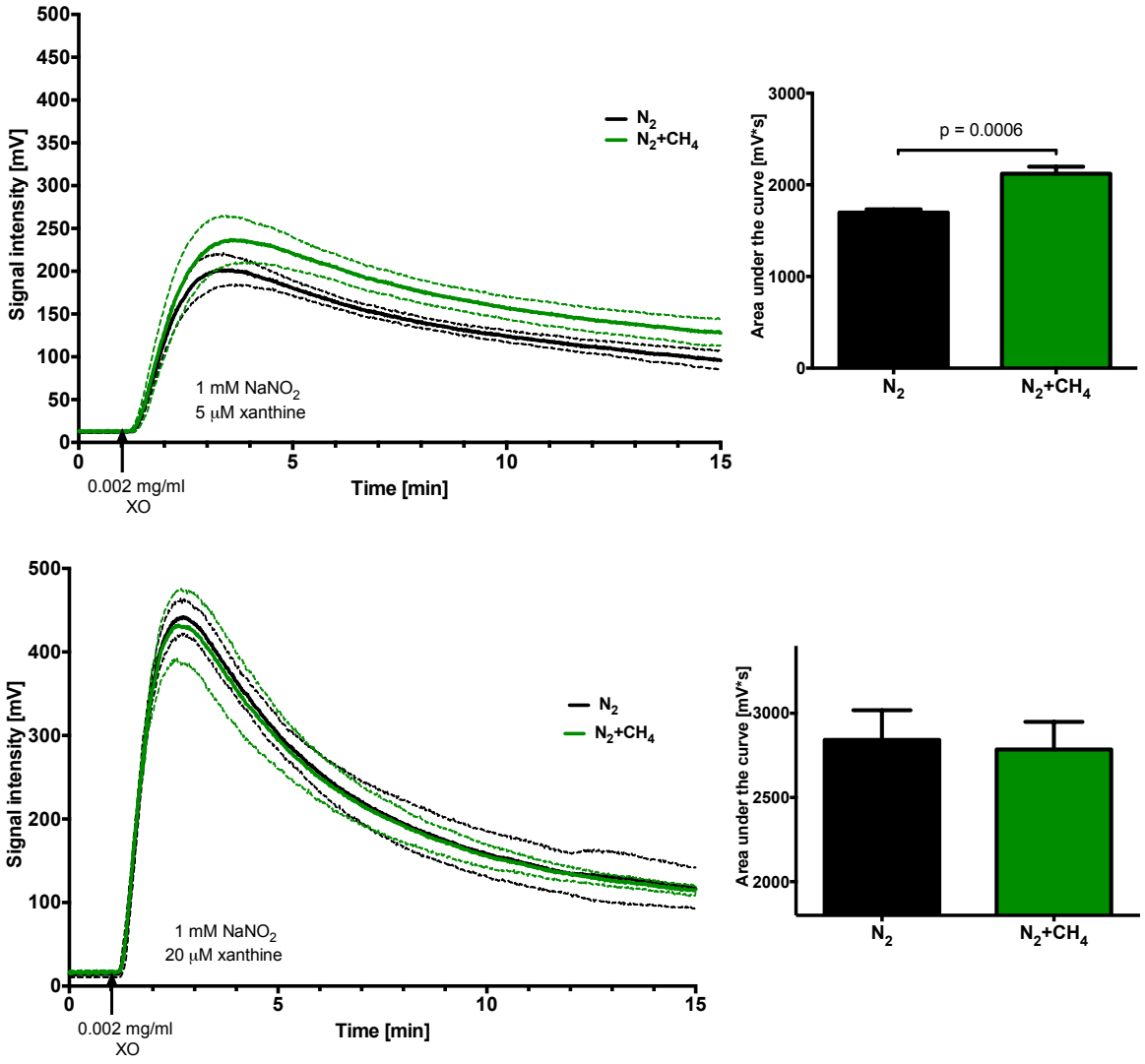


Figure 14 Effect of CH_4 on NO produced by a XOR-xanthine enzyme system
(A) NO generated by XOR was significantly increased (p = 0.0006; assessed by two-tailed, unpaired t-test) in the presence of methane when supplying 5 µM xanthine as an electron acceptor.
(B) However, with increasing xanthine concentrations, this effect was reduced and at 20 µM xanthine, no significant effect of CH_4 on the NO production was observed.

When exchanging the substrate xanthine for another substrate of XOR, NADH, which acts at the FAD site of the enzyme, only a very slight NO production by the enzyme could be measured (Figure 15). In the presence of CH_4, a small trend of increased NO production was observed but failed to show any significance when compared to the N_2 control measurements.

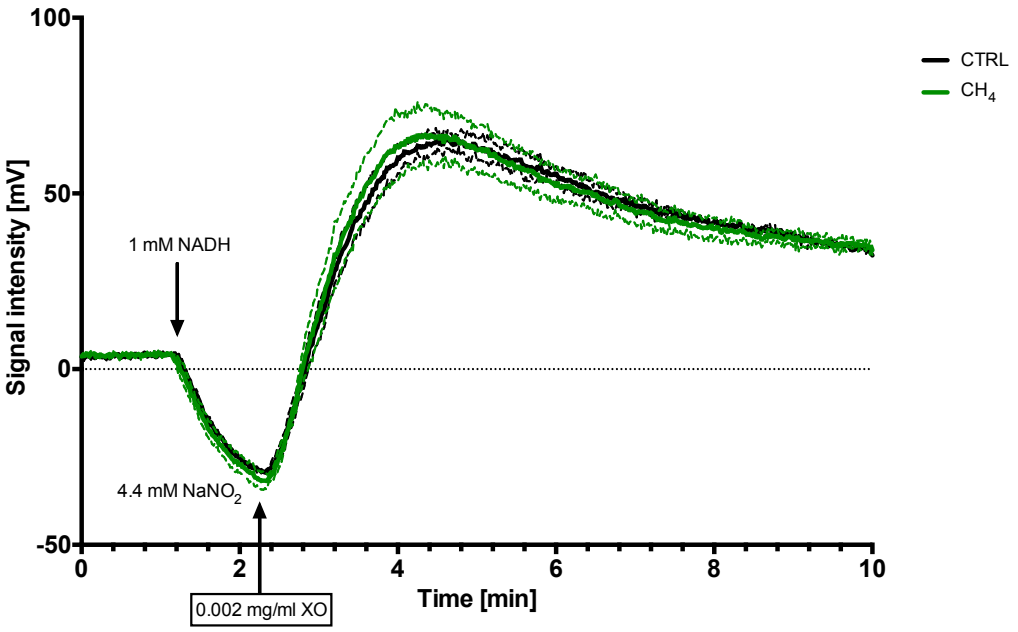


Figure 15 Effect of CH_4 on NO produced by a XOR-NADH system
NO generation by 0.002 mg/mL XOR using 1 mM of NADH as an electron acceptor substrate only yielded a small NO signal from the reduction of 4.4 mM sodium nitrite. In measurements containing methane in the carrier gas mixture, this NO production showed a small trend towards an increase of the signal but failed to show significance.

Considering that the effect of CH_4 in the intestine can be due to specific patterns of substrates in tissue we repeated the experiment in intestinal homogenate. To verify these results, the experiments of the effect on CH_4 on NO production were repeated on intestine homogenate samples. However, the NO production by intestine homogenate was slightly but significantly reduced ($p = 0.009$) in samples equilibrated in carrier gas containing 2.2 % CH_4 as can be seen in Figure 16.

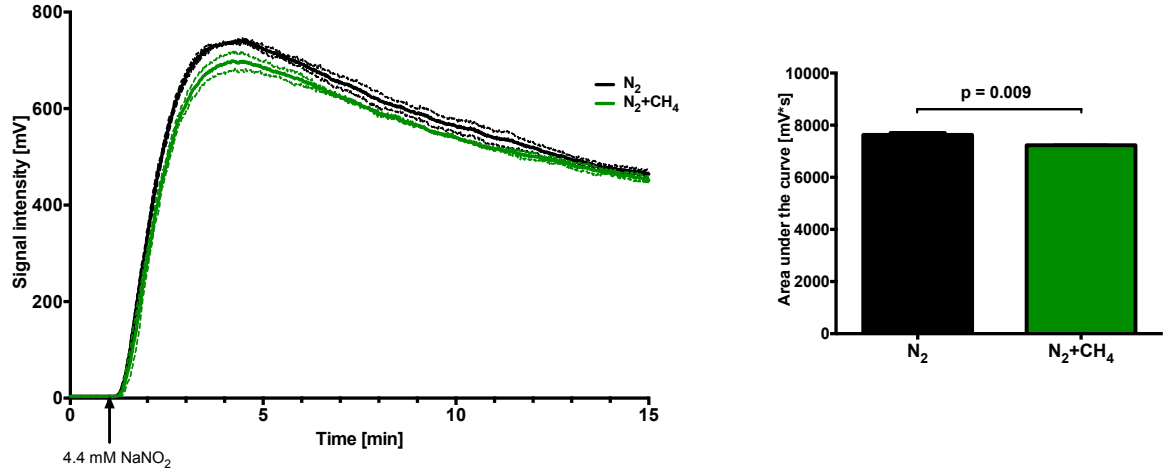


Figure 16 Effect of CH_4 on the NO generation in intestine homogenate
NO production in intestine homogenates stimulated by the addition of 4.4 mM sodium nitrite was slightly but significantly reduced ($p = 0.009$; assessed by two-tailed, unpaired t-test) when using a nitrogen carrier gas containing 2.2 % CH_4 in comparison to pure N_2 as a control.

In order to rule out a possible CH_4-induced increased scavenging of NO by the homogenate as a possible explanation of the reduction of NO release during the homogenate measurements, the NO donor diethylenetriamine NONOate (DETA NONOate) was added to

homogenate measurements instead of nitrite, to provide an independent and constant source of NO. In these measurements, virtually no change was observed between methane and N_2 control measurements, showing no increase in NO scavenging in the presence of methane, as can be seen in Figure 17.

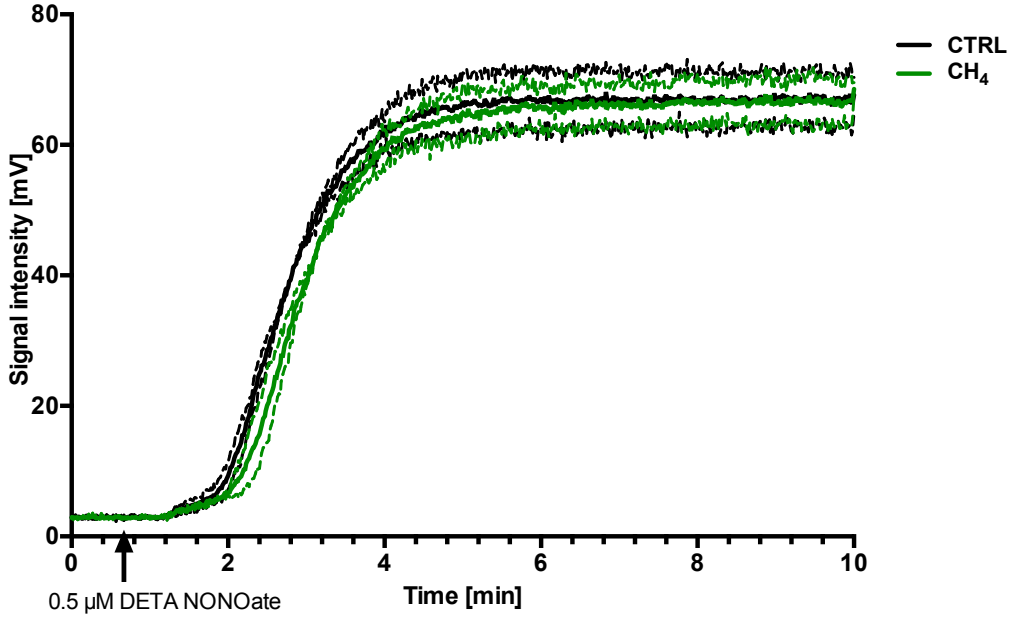


Figure 17 Investigation of a possible increase in NO scavenging due to CH_4 presence
Adding the independent NO donor DETA NONOate to intestine homogenate samples instead of using nitrite to stimulate endogenous NO production via nitrite reduction showed near-identical signals both using the methane-containing gas mixture and pure N_2 carrier gas.

Theorizing that the effect of methane seems to be dependent on the types and ratios of substrates present for nitrite reduction by XOR, the substrate composition of intestine homogenate was to be mimicked in measurements of the enzyme system to achieve comparability between both measurement types. Therefore, an aliquot of intestine homogenate was boiled to denature and therefore deactivate any enzymes present, before equilibration in the atmosphere of N_2 containing 2.2 % CH_4. With a half-life at 100 °C of approximately 10 minutes, NAD^+ is the most thermally instable substrate of XOR. Boiling time of the homogenate was therefore set to 5 minutes to ensure denaturation of the enzymes while maintaining at least 75 % of the original substrate concentrations.[81] Sodium nitrite (1 mM) was added at the beginning of the measurement to provide the basis for nitrite reduction, which led to a slight but constant elevation of the signal baseline. After the introduction of XOR, however, a similar signal to previous homogenate measurements was observed. In these measurements designed to shed light on the effect of methane on XOR using substrate ratios similar to what is found in intestine homogenate. In this experiment, the situation observed in the ischemic intestine *in vivo* was reproduced *in vitro*.[80] The presence of CH_4 significantly decreased XOR-mediated NO production when compared to a N_2 control ($p = 0.023$ according to a two-tailed unpaired t-test, Figure 18).

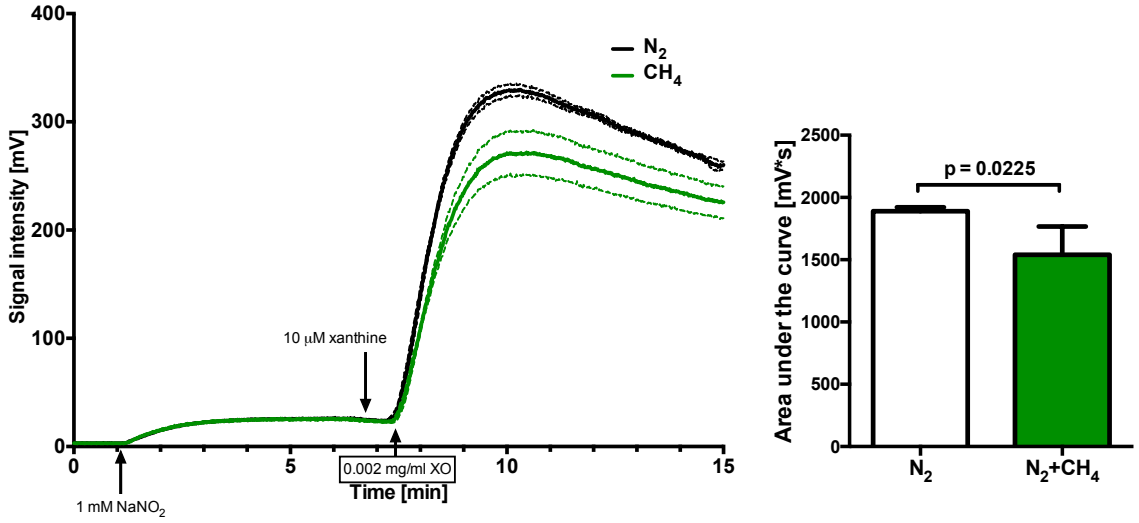


Figure 18 Mimicking the substrate ratios in intestine homogenate for pure XOR measurements
After the addition of 1 mM sodium nitrite, a small but constant increase of the baseline was observed. Only upon adding the pure enzyme (0.002 mg/mL), however, is a significant amount of NO produced from the substrates present in the denatured intestine homogenate sample. In measurements using the CH_4-N_2 gas mixture, the overall amount of NO is significantly reduced (p = 0.0025, two-tailed, unpaired t-test) when compared to pure N_2 control runs.

To further investigate this effect, these samples combining denatured intestine homogenate and isolated XOR enzyme were incubated with the FAD-site inhibitor diphenyleneiodonium chloride (DPI) at a concentration of 100 mM for 10 minutes before starting the measurements. As both NAD^+ and NADH act on the FAD site of the enzyme, this was theorized to only leave nitrite, xanthine and hypoxanthine as eligible substrates for NO reduction as they act on the still-functional Molybdenum site. In the presence of methane, the thus treated samples exhibited a higher NO release than control measurements without CH_4, similar to the effect of methane observed in a pure XOR system containing only xanthine and nitrite as substrates, shown in Figure 19. This suggests that FAD-side of XOR is important for the *in vivo* effects of CH_4.

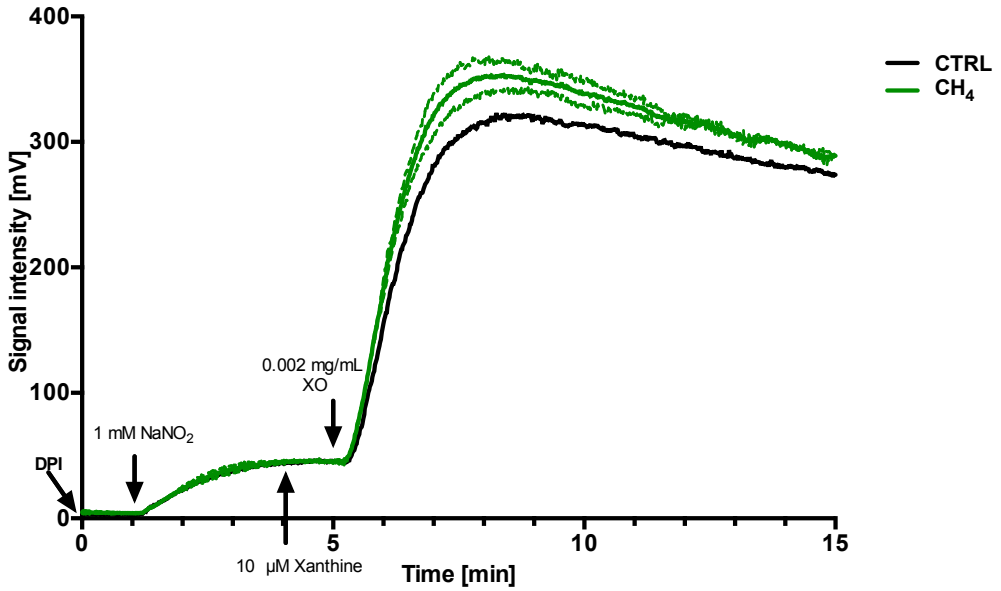


Figure 19 Effect of DPI on NO production by XOR from substrate ratios in intestine
Treating the denatured intestine homogenate with the FAD-site inhibitor FAD showed to reverse the increasing effect of CH_4 on the NO production to an increase in NO released from the samples in the presence of methane.

4. Discussion

In the first part of this project, we have characterized the kinetics of nitrite reduction to NO and different organs, but we could not explain differences in the NO release in different organs. The second part of the study was aimed at understanding the mechanisms underlying the kinetics of NO release, and in particular, the origin of lag-phase observed in NO release. In the last part we identified the major generator of NO in tge intestine and its regulation by methane, a gaseous messenger recently discovered in mammalians. The nature of the nitric oxide production in liver homogenates from nitrite by different nitrite reductases was investigated and characterized using a continuous chemiluminescence NO detection method. The time period between addition of $NaNO_2$ for nitrite reduction and the start of the bulk release from the homogenate samples, titled the lag phase, was found to be strongly proportional to the concentration of nitrite used for the measurement, ranging from about 4 minutes at 2.1 mM sodium nitrite to approximately 14 minutes at nitrite concentrations of 15.6 mM (Figure 7).

In a paper previously published in our laboratory, we showed that increased oxygen concentration oxidizes NO and consequently slows down the formation of –SNO complexes and increase the duration of the lag-phase. Additionally, our experiments have shown that the lag phase observed in nitrite reduction is due to initial binding of produced NO to compounds in the homogenate, such as the known NO scavenger hemoglobin or formation of S-nitrosothiols from NO and thiol groups in proteins. The increased NO production from larger amounts of nitrite would then saturate the available bonds for NO scavenging faster, thereby leading to reduced lag phases. An initial attempt to test this theory by blocking thiol groups from forming -SNO by means of a mixture of NEM and EDTA showed to be incompatible with the NOA method as the reagents interfered with the measurement; however, pre-treatment of homogenates with different aliquots of a saturated solution of NO in bi-distilled water managed to proportionally reduce the lag time from 14 min in the control down to immediate release of bulk NO at an addition of 300 µL saturated NO solution. Magnitude and slope of the NO signal curve were conserved through in all measurements, irrespective of the addition of NO solution, suggesting that the lag phase is indeed caused by initial NO scavenging (Figure 9). The contribution of NO-Hb binding to the lag phase was then allocated by adding a perfusion step in the tissue harvesting process. Prior to excision of liver tissue, the rat liver was perfused using Ringer solution containing heparin via the *Vena hepatica* until a uniform light pink color indicated the removal of blood from the tissue. Measurements from the thus collected tissue exhibited a much-reduced lag phase from the 14 min of the control measurements of non-perfused liver tissue to about 2 min in the perfused sample, as is shown in Figure 10. This gives rise to the conclusion that the lag phase observed is predominantly due to the scavenging of NO by hemoglobin. The remaining two-minute delay observed in the measurement using perfused liver homogenate

was then attributed to a failure to completely remove all blood from the tissue and the binding of NO to other compounds, e.g. via the formation of -SNO.

Upon replication of these measurements using homogenates from different tissues, such as rat heart and intestine, the non-existent lag time in the less blood retaining intestine tissue and the prominent lag time in well-vascularized tissues, e.g. liver, were in line with the previous conclusion that the lag time seems to be predominantly caused by NO-Hb bond formation.

As the method was to be characterized for future use as a model for ischemic conditions, the more physiologically relevant measurement conditions of 37 °C and the mildly acidic pH 6.0 were compared to the previously used 22 °C and pH 7.4. Both raising the temperature and lowering the pH to these more relevant parameters for ischemia led to a strong increase of the existing NR activity, furthering the role of nitrite reductases as the main contributors to hypoxic NO production.

As a third step in the project, the source of the NO measured under ischemic conditions was identified in intestinal tissue. For this purpose, the contributions of certain nitrite reductases were to be assessed by either using specific inhibitors on the homogenates and comparing its NO release with untreated samples or measuring the individual NO production capabilities of isolated nitrite reductases. For the inhibition of nitrite reductases in the tissue samples, the homogenate was treated with allopurinol, an inhibitor of xanthine oxidoreductase, or myxothiazol, an inhibitor of cytochrome bc1, the part of the mitochondrial respiratory chain previously attributed with NR activity. While myxothiazol had to be used at a concentration of 30 µM in order to achieve inhibition, allopurinol showed near-complete inhibition of NO production at both 100 and 150 µM, placing the contribution of xanthine oxidoreductase greatly over the NR activity of mitochondria. In measurements supplying whole blood containing heparin as an anticoagulant instead of homogenate to investigate the NR activity of hemoglobin, the NO signal failed to rise significantly from baseline levels, suggesting that the NO scavenging capabilities of NO dominate over its activity as a nitrite reductase under ischemic conditions. Using previously deoxygenated myoglobin from horse heart as an isolated nitrite reductase, only a small NO signal was observed even at a nitrite concentration as high as 15 mM. This NO production by myoglobin was inhibited by the xanthine oxidoreductase inhibitor allopurinol, hinting at an off-target effect of the inhibitor on the nitrite reductase. As the overall contribution of myoglobin on the NO production at physiological concentrations is very small, especially in intestine and liver tissue, however, xanthine oxidoreductase was concluded to be the main nitrite reductase and the major source of NO production under ischemic conditions.

Replacing the more complex tissue homogenate with a pure enzyme system posed challenges in itself, xanthine oxidoreductase being an enzyme with two different domains available for binding to various substrates, a molybdenum site where both the nitrite reduction and the oxidation of hypoxanthine or xanthine take place, and a flavin adenine dinucleotide (FAD) site able to react with NADH, NAD^+ and oxygen, the latter of which being less relevant in a hypoxic context. Both the molybdenum and FAD site are connected via a FeS cluster that allows for electron transfer in the enzyme. The occurrence of multiple

possible substrates makes the exact comparison of the xanthine oxidoreductase system with homogenate measurements challenging, but measurements supplying physiological concentrations of 5 – 20 µM xanthine or hypoxanthine as a substrate in addition to 1 mM sodium nitrite for NO production showed a signal very similar to the one produced by intestine homogenate in appearance, slope and magnitude.

To investigate a suspected interaction between the gasotransmitter methane and the NO metabolism, the xanthine oxidoreductase measurements were repeated replacing the pure N_2 carrier gas with a gas mixture containing 2.2 % CH_4 in nitrogen. As with the N_2 control measurements, the samples were thoroughly mixed by bubbling the carrier gas through the liquid in the reaction chamber, thereby not only purging any produced NO from the sample and into the detection unit but also ensuring contact of CH_4 with the reaction mixture. During initial experiments measuring the NO production by 0.002 mg/mL xanthine oxidoreductase from 5 µM xanthine and 1 mM $NaNO_2$, CH_4 was shown to increase the NO release from the sample. However, this effect lessened with increasing concentrations of xanthine and disappeared altogether at 20 µM xanthine. Replacing the xanthine with 1 mM NADH decreased the rate of NO release and only a slight trend towards an increase in NO in the presence of CH_4 could be observed. To verify an effect of CH_4 on the tissue NO production, the measurements of NO from intestine homogenates supplying 4.4 mM $NaNO_2$ were repeated in the presence of CH_4, which had a contrasting effect of a significant decrease ($p = 0.009$) of the overall NO produced. To rule out that the observed decrease in NO release is caused by an increased scavenging of NO and not actually due to an effect of methane on the enzyme activity, an experiment was set up equilibrating tissue homogenate under either CH_4 or N_2 gas atmosphere. After 10 min of equilibration, the constant NO donor DETA NONOate was then added to supply an independent source of NO. As measurements under methane and nitrogen yielded virtually identical curves, an increased scavenging of NO in the tissue due to the presence of methane was dismissed. Another theory as to the cause of the discrepancy between the increasing effect of CH_4 on a pure xanthine oxidoreductase system using xanthine as a substrate, and the decreasing effect of CH_4 in tissue homogenate, attributed it to the stark difference in available substrates in both systems. While the enzymes in tissue homogenates have all possible substrates in their physiological ratios available for reactions, the isolated enzyme system only supplies xanthine or NADH at somewhat arbitrary concentrations. So in order to create an environment of original substrate ratios and concentrations for the pure enzyme system, an aliquot of tissue homogenate was boiled to denature any present enzymes. The thus deactivated homogenate was then used as a substrate mixture and equilibrated under either CH_4 or pure N_2 gas. Supplying 1 mM $NaNO_2$ a slight but steady increase in baseline NO detection was measured, but only upon the addition of 0.002 mg/mL xanthine oxidoreductase was the bulk release of NO induced. In these measurements, the presence of CH_4 led to a decrease in NO production by xanthine oxidoreductase, similar to the effect observed in intestine homogenates. This suggests that the effect of methane on the NO production might be due to a direct action of CH_4 on the xanthine oxidoreductase enzyme. In order to further characterize this action of CH_4 on XOR, the FAD-site inhibitor was added to the deactivated homogenate prior to the measurement and 1 mM of sodium nitrite was supplied for nitrite reduction. Upon addition of pure xanthine

oxidoreductase, an increasing effect of methane on the NO production, similar to the effect of methane on XOR using only xanthine as a substrate, was shown. To sum up these last findings, methane shows an increasing effect on the NO production by xanthine oxidoreductase when only substrates acting at the molybdenum site, e.g. xanthine, hypoxanthine, and nitrite, are available or when the FAD site has been rendered inactive by use of an inhibitor, and a decreasing effect on the NO release from the sample when it has access to all substrates present in the tissue.

The effect of methane, therefore, seems to be strongly dependent on the substrate concentrations and ratios at the site of the tissue.

5. Conclusion

The key conclusions of this project are that the kinetics of NO release from nitrite is characterized in different tissues; we have shown that NO initially produced from nitrite is scavenged mainly by Hb to form Hb-NO and possibly by –SH groups, causing a lag phase in NO release observed in tissue homogenates. Under hypoxic conditions NO production is predominantly catalyzed by the xanthine oxidoreductase under ischemic conditions; cytochrome bc1 and myoglobin contribute as well but to a lesser extent. And lastly, the presence of methane has a measurable impact on NO production in ischemic tissue, the direction of which depends on the ratio and types of substrates of xanthine oxidoreductase present. Under the concentrations present in the intestine, methane significantly decreases NO production from nitrite reduction.

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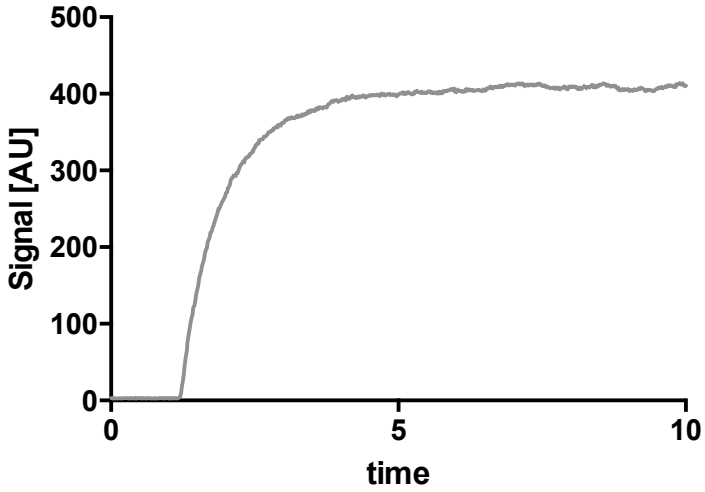
7. Appendices

Supplementary Figures



Supplementary Figure 1 NO release from NEM in incubation buffer
Control experiment of 20 µM NEM in incubation buffer without addition of nitrite or homogenate shows that NEM acts as a NO donor in the system.

List of figures

List of abbreviations

ADP	adenosine diphosphate
allo	Allopurinol
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
CH_4	methane
CO	carbon monoxide
CO-Hb	carboxyhemoglobin
CTRL	control
DETA-NONOate	diethylenetriamine NONOate
deoxyhemoglobin	deoxyHb
deoxymyoglobin	deoxyMb
DPI	diphenyleneiodonium chloride
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
Hb	hemoglobin
HO-1/2	heme oxygenase-1/2
H_2S	hydrogen sulfide
Mb	myoglobin
methemoglobin	metHb
metmyoglobin	metMb
min	minute(s)
myxo	myxothiazol
N_2	nitrogen
NADH, NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NO	nitric oxide
NO-Hb	nitrosylated hemoglobin
NOA	Nitric Oxide Analyzer, Sievers 280i (General Electrics)
NR	nitrite reductase(s)
RNS	reactive nitrogen species
ROS	reactive oxygen species
-SNO	S-nitrosothiol
SNO-Hb	S-nitrosohemoglobin
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase