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NADPH OXIDASE AND XANTHINE OXIDOREDUCTASE AS TARGETS AND REGULATORS OF THE NITRATE-NITRITE-NITRIC OXIDE PATHWAY

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NADPH Oxidase and Xanthine Oxidoreductase as Targets and Regulators of the Nitrate-Nitrite-Nitric Oxide Pathway

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To my parents,

Στους γονείς μου,

Beauty lies in simplicity:

Consider how the lilies of the field grow: They do not labor or spin. Yet, I tell you that not even king Solomon in all his glory was adorned like one of these.
(Matthew, 6:28-29)

LIST OF SCIENTIFIC PAPERS

I. Gao X, Yang T, Liu M, PELELI M, Zollbrecht C, Weitzberg E, Lundberg JO, Persson AE, Carlström M.

NADPH oxidase in the renal microvasculature is a primary target for blood pressure-lowering effects by inorganic nitrate and nitrite. *Hypertension. 2015 Jan ; 65(1):161-70*

II. Yang T*, PELELI M*, Zollbrecht C, Giulietti A, Terrando N, Lundberg JO, Weitzberg E, Carlström M.

Inorganic nitrite attenuates NADPH oxidase-derived superoxide generation in activated macrophages via a nitric oxide dependent mechanism.
*Free Radic Biol Med. 2015 Jun;83:159-66, * equal contribution*

III. PELELI M*, Hezel M*, Zollbrecht C*, Persson AE, Lundberg JO, Weitzberg E, Fredholm BB, Carlström M.

In adenosine A_{2B} knockouts acute treatment with inorganic nitrate improves glucose disposal, oxidative stress and AMPK signaling in the liver.
*Front Physiol. 2015 Aug 7;6:222, * equal contribution*

IV. Hezel M*#, PELELI M*, Liu M*, Zollebrecht C*, Jensen B, Checa A, Giulietti A, Wheelock C, Lundberg JO, Weitzberg E, Carlström M#

Dietary nitrate improves age-related hypertension and metabolic abnormalities via modulation of angiotensin II receptor signaling and inhibition of superoxide generation.
*Free Radic Biol Med., 2016 Jul 26;99:87-98. *equal contribution, # corresponding authors*

V. PELELI M, Zollbrecht C, Montenegro MF, Hezel M, Zhong J, Persson E.G, Holmdahl R, Weitzberg E, Lundberg JO# and Carlström M#.

Enhanced XOR activity in eNOS-deficient mice: Effects on the nitrate-nitrite-NO pathway and ROS homeostasis.
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ADDITIONAL PUBLICATIONS

Other published papers involving the author that are not included in the thesis (chronological order):

I. Schiffer TA, **Peleli M**, Sundqvist ML, Ekblom B, Lundberg JO, Weitzberg E, Larsen FJ.
Control of human energy expenditure by cytochrome c oxidase subunit IV-2. Am *J Physiol Cell Physiol. 2016 Sep 1;311(3):C452-61*.

II. Porpino SK, Zollbrecht C*, **Peleli M***, Montenegro MF, Brandão MC, Athayde-Filho PF, França-Silva MS, Larsson E, Lundberg JO, Weitzberg E, Persson EG, Braga VA, Carlström M.
Nitric oxide generation by the organic nitrate NDBP attenuates oxidative stress and angiotensin II-mediated hypertension.
Br J Pharmacol. 2016 Jul;173(14):2290-302.*equal contribution

III. **Peleli M***, Al-Mashhadi A*, Yang T, Larsson E, Wåhlin N, Jensen BL, G Persson AE, Carlström M.
Renal denervation attenuates NADPH oxidase-mediated oxidative stress and hypertension in rats with hydronephrosis.
*Am J Physiol Renal Physiol. 2016 Jan.*equal contribution*

IV. Yang T, Gao X, Sandberg M, Zollbrecht C, Zhang XM, Hezel M, Liu M, **Peleli M**, Lai EY, Harris RA, Persson AE, Fredholm BB, Jansson L, Carlström M.
Abrogation of adenosine A1 receptor signalling improves metabolic regulation in mice by modulating oxidative stress and inflammatory responses.
Diabetologia. 2015 Jul;58(7):1610-20.

V. Carlström M, Liu M, Yang T, Zollbrecht C, Huang L, **Peleli M**, Borniquel S, Kishikawa H, Hezel M, Persson AE, Weitzberg E, Lundberg JO.
Cross-talk Between Nitrate-Nitrite-NO and NO Synthase Pathways in Control of Vascular NO Homeostasis.
Antioxid Redox Signal. 2015 Aug 1;23(4):295-306.

VI. Liu M, Zollbrecht C, **Peleli M**, Lundberg JO, Weitzberg E, Carlström M. Nitrite-mediated renal vasodilatation is increased during ischemic conditions via cGMP-independent signaling. *Free Radic Biol Med. 2015 Jul;84:154-60.*

VII. **Peleli M***, Aggeli IK*, Matralis AN, Kourounakis AP, Beis I, Gaitanaki C. Evaluation of two novel antioxidants with differential effects on curcumin-induced apoptosis in C2 skeletal myoblasts; involvement of JNKs. *Bioorg Med Chem. 2015 Feb 1;23(3):390-400.*equal contribution*

VIII. Gao X*, **Peleli M***, Zollbrecht C, Patzak A, Persson AE, Carlström M.
Adenosine A1 receptor-dependent and independent pathways in modulating renal vascular responses to angiotensin II.
Acta Physiol (Oxf). 2015 Jan;213(1):268-76. equal contribution*

ABSTRACT

Overproduction of reactive oxygen species (ROS) by NADPH oxidase (NOX) and xanthine oxidoreductase (XOR) with a concomitant decrease in the bioavailability of nitric oxide (NO) from eNOS contributes to the development of cardiovascular and metabolic disorders. Extensive research has proven the existence of an alternative NOS-independent pathway for NO production. This nitrate-nitrite-NO pathway starts with inorganic nitrate which is derived from the diet and from oxidized NO. Nitrate is reduced to nitrite mainly by oral commensal bacteria and then to NO and other bioactive nitrogen oxides in blood and tissues.

The aim of the current thesis was to investigate the therapeutic role of the nitrate-nitrite-NO pathway in models of hypertension, metabolic dysfunction and inflammation focusing on whether the main ROS-producing enzymes NOX and XOR could be targets or even regulators of the this pathway. We show that NOX in the renal microvasculature is a primary target for the blood pressure lowering effects of dietary nitrate in Angiotensin-II (AngII)-mediated hypertension. In agreement, NOX activity and AngII-induced receptor signaling are downregulated by nitrate in aged and hypertensive rats. Moreover, nitrate targets the elevated liver NOX activity of aged and metabolically dysregulated mice with an improvement of AMPK activity. Finally, we show that nitrite can act on cells of the innate immune response. In particular, NOX mediated superoxide production is strongly attenuated in activated macrophages with a concomitant reduction on iNOS gene expression and peroxynitrite production. Mechanistically, we observed that symbiotic bacteria and XOR together are responsible for the bioactivation of dietary nitrate to form NO. In addition, the effects of nitrate and nitrite are not only NOS independent but actually potentiated when eNOS activity is pharmacologically, genetically or naturally impaired. Interestingly, the absence of eNOS is associated with a higher XOR activity that partly compensates for the disrupted NO homeostasis and elevated blood pressure in these mice. Also, the blood pressure response to dietary nitrate is augmented in $eNOS^{-/-}$ mice and abolished upon XOR inhibition. Interestingly, nitrate and nitrite were able to switch the function of XOR towards lower ROS and higher NO production which could significantly contribute to the antihypertensive effects.

In conclusion, administration of nitrate or nitrite is associated with a number of therapeutic cardiovascular and metabolic effects in animal models of disease. We propose that NOX and XOR are two main targets and possible regulators of the nitrate-nitrite-NO pathway. This pathway is triggered in situations with higher NOX and XOR activity and acts in parallel with the NOS dependent NO production to uphold NO homeostasis.

CONTENTS

LIST OF ABBREVIATIONS

A_1	Adenosine type 1 receptor
A_{2A}	Adenosine type 2A receptor
A_{2B}	Adenosine type 2B receptor
$A_{2B}^{-/-}$	Adenosine type 2B receptor knockout mice
A_3	Adenosine type 3 receptor
ACE	Angiotensin II converting enzyme
Ach	Acetylcholine
AICAR	Aminoimidazole carboxamide ribonucleotide
AMPK	5' adenosine monophosphate-activated protein kinase
AngI	Angiotensin I
AngII	Angiotensin II
AQP	Aquaporin
AT_1	Angiotenin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
cPTIO	Carboxy-PTIO potassium salt
DAF-FM	4-Amino-5-methylamino-2′,7′ difluorofluorescein
DAG	Diacylglycerol
DETA-NONOate	Diethylentriamine/NO adduct
DHE	4-amino-5-methylamino-2’,7’-difluorescein diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DUOX 1	Dual oxidase 1
DUOX 2	Dual oxidase 2
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
$eNOS^{-/-}$	Endothelial nitric oxide synthase knockout mice
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
Febuxostat	2-(3-cyano-4-isobutoxyphenyl)-4-methyl-1,3-thiazole-5-carboxylic acid
GLUT-4	Glucose transporter 4
GTP	Guanosine triphosphate
HEPES	Hydroxyethyl piperazine ethane sulfonic acid
HFD	High fat diet
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HNO	Nitroxyl
HOMA-IR	Homeostatic model assessment insulin resistance
HPF	Hydroxyphenyl fluorescein
HRP	Horseradish peroxidase
IBMX	3-Isobutyl-1-methylxanthine
iNOS	Inducible nitric oxide synthase
$iNOS^{-/-}$	Inducible nitric oxide synthase knockout mice
i.p	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IRI	Ischemia reperfusion injury
IRS-2	Insulin receptor 2

L-arg	L-arginine
L-cit	L-citrulline
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
MPO	Myeloperoxidase
mRNA	Messanger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
$nNOS^{-/-}$	Neuronal nitric oxide synthase knockout mice
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
NSAID	Non-steroidal anti-inflammatory drug
O_2^-	Superoxide anion
Oxypurinol	1,2-Dihydropyrazolo[4,3-e]pyrimidine-4,6-dione
PARP	Poly [ADP-ribose] polymerase
PBS	Phosphate-buffered saline
PG-VSMC	Preglomerular vascular smooth muscle
PKC	Protein kinase C
phox	Phagocytic oxidase
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
RAS	Renin angiotensin II system
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium

sGC	Soluble guanyl cyclase
$SOD1^{-/-}$	Superoxide dismutase 1 knockout mice
SOD 2	Superoxide dismutase 2
TBS-T	Tris-buffered saline-Tween
VSMC	Vascular smooth muscle cells
wt	wild type
Xan	Xanthine
XDH	Xanthine Dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

1 INTRODUCTION

1.1 Reactive oxygen species and the NADPH oxidase family

Reactive oxygen species (ROS) are chemical units which belong to two main categories: a) free radicals (e.g superoxide, hydroxyl radical) and b) non-radicals (e.g hydrogen peroxide). These small molecules serve as signaling messengers when they are produced at low levels and have crucial physiological roles in host defense, hormone biosynthesis, synaptic plasticity etc. However, it is also well known that higher levels of ROS increase overall oxidative stress, and contribute to the progression and the pathology of cardiovascular and metabolic disease [1, 2]. Mitochondria [3], xanthine oxidoreductase (XOR) [4], NADPH oxidases (NOX) [5] and uncoupled endothelial NO synthase (eNOS) [6] are considered the major cellular sources of ROS. Among them, the enzymatic family of NOX is suggested to play a key role in the pathophysiology of many cardiovascular and metabolic complications [5].

NOX is a multi-subunit enzyme that catalyzes the reduction of oxygen to superoxide by transferring one electron from NADPH. The family consists of seven members, which are named NOX1, NOX2 (or gp91phox), NOX3, NOX4, NOX5 (only in humans), Dual oxidase 1 and 2 (DUOX1, DUOX2) [7]. NOX1 is found in colon and vascular cells and plays a role in host defense and cell growth, NOX2 is the catalytic subunit of the respiratory burst in phagocytes, but is also expressed in vascular, cardiac, renal, and neural cells and NOX4, also termed as renal oxidase (RENOX) because of its high abundance in the kidney, is found in vascular cells and osteoclasts [8]. NOX2, together with its subunits p47phox, p67phox, p40phox, p22phox, is the prototypical and best characterized NOX. In non-stimulated cells p47phox, p67phox and p40phox remain in the cytosol while p22phox and gp91phox are constitutively expressed on the cell membrane. Upon stimulation, p47phox is phosphorylated and the three cytosolic subunits form a complex and translocate to the membrane where they associate with the two other membrane subunits and create the fully activated enzyme. In this activation cascade, the small GTP binding protein Ras-related C3 botulinum toxin substrate Rac1 also plays an important role (detailed illustration of the assembly process in fig.1). Although NOX2 has been well characterized it is still not fully understood how the rest of the NOX family members are regulated. All of them seem to require p22phox for their activity, but not necessarily all or the same cytosolic subunits as for NOX2. Moreover, NOX4 is constitutively active in the endothelium and the vascular smooth muscle cells (VSMC) can produce large amounts of hydrogen peroxide apart from superoxide [9].

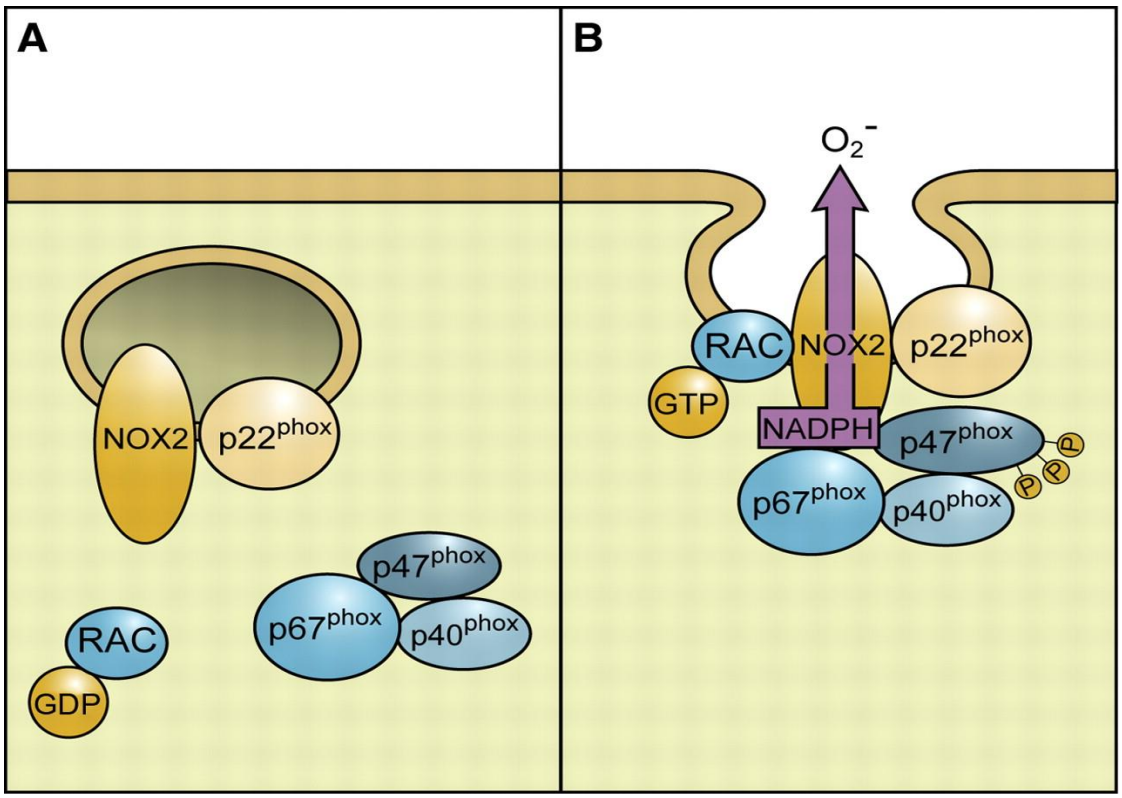

Figure 1: *Schematic illustration of the assembly process of the phagocytic/prototypical NOX. In non-activated cells, NOX2 (or gp91phox) and p22phox are expressed in the membrane of intracellular vesicles. In activated cells, GDP is exchanged for GTP on Rac allowing its translocation to the vesicle membrane. Moreover, phosphorylation of the cytosolic p47phox subunit allows it to interact with p22phox. The movement of p47phox brings with it the other cytoplasmic subunits, p67phox and p40phox, to form the final active NOX2 enzyme complex. The NOX2-containing vesicles fuse then with the plasma membrane and electrons are transported from cytoplasmic NADPH to oxygen leading to superoxide production(image from Bedard K.et al. 2007 [5]).*

1.1.1 NADPH oxidases, AngII-mediated signaling and cardiovascular disease

In several animal models it has been shown that NOX-derived ROS are crucial for the development of cardiovascular disease including hypertension [10]. In particular, AngII-dependent hypertension seems to be coupled to NOX-induced oxidative stress. AngII is a peptide derived from a cascade of proteolytic events where renin produced in the kidney cleaves angiotensinogen to AngI and AngI is subsequently cleaved to AngII by the AngII converting enzyme (ACE) [11]. AngII is a very potent vasoconstrictor closely linked to NOX-mediated ROS production, resulting in increased arterial blood pressure [12, 13]. For example, AngII infusion in rats promotes elevated expression and activity of NOXs [14]. However, in mice and rats genetically ablated for p47phox, NOX2 or p22phox AngII induced hypertension is attenuated [15-18]. Apart from the experimental studies, clinical data show a link between NOX-derived ROS and cardiovascular disease. It has been shown that there is increased myocardial NOX activity during heart failure, and hypertensive patients with genetic predisposition for increased p22phox expression have a more aggravated pathologic phenotype [19, 20]. Moreover, lymphoblasts from hypertensive patients present higher p22phox expression and ROS production [21]. In addition, pre-eclampsia has been associated with the production of agonistic autoantibodies that target the AngII type 1 (AT_1) receptor and strongly activate NOX activity and expression [22]. In general, the importance of the AT_1

mediated receptor signaling and NOX-derived ROS is supported by clinical studies where they report that among the major anti-hypertensive drugs, the ACE inhibitors and the AT_1 receptor blockers are much more potent in improving vascular structure and function and simultaneously reducing oxidative stress [23-25].

It is important to emphasize that oxidative stress and in particular NOX-derived superoxide are also increased and involved in several other models of hypertension which are AngII independent such as the spontaneously hypertensive rats (SHR), DOCA-salt hypertension and Dahl salt-sensitive rat [26-29].

1.1.2 NADPH oxidases and metabolic syndrome

The metabolic syndrome, a cluster of risk factors including insulin resistance, hyperinsulinemia, impaired glucose tolerance, dyslipidemia, obesity, and elevated blood pressure, has reached epidemic proportions in industrialized countries [30]. The past few years have also increased the recognition of its frequent association with cardiovascular disease, especially atherosclerosis, which constitutes a major cause of morbidity and mortality in individuals with metabolic syndrome [31]. Oxidative stress, and particularly superoxide, is considered to be an important contributor in the pathogenesis of hypertension, hypertriglyceridemia, diabetes, and obesity [32].

From a clinical perspective, it has been shown that NOX overactivity in peripheral phagocytic mononuclear cells underlies oxidative stress in patients with metabolic syndrome [33]. First of all, the hyperactivation of NOXs has been linked to reduced insulin production. For example, chronic hyperglycemia inhibits insulin secretion, activates AT_1 receptor, and increases superoxide production and p47phox and p22phox expression in an insulin producing rat cell line and in isolated human pancreatic islets [34-36]. Apart from the pancreas, increased activity and expression of the NOXs in other metabolic organs seem to play a detrimental role in the progression of metabolic syndrome and the eventual development of type 2 diabetes. Although the mechanistic details have not been clarified yet, there are many studies showing that the NOX family in the liver plays a critical role in the development of metabolic syndrome and diabetes [37]. Moreover, AngII-induced NOX activation impairs insulin signaling and glucose uptake in skeletal muscle via reduced GLUT-4 transporter translocation [38].

1.2 The biological functions of nitric oxide

NO is a key signaling messenger in the cardiovascular system [39]. Impaired NO metabolism, bioavailability and production are considered important risk factors for the development or progression of cardiovascular disease, chronic kidney disease, metabolic syndrome and type 2 diabetes [40, 41] . In addition to its role as an endothelium-derived relaxing factor, NO serves many important biological functions in cardiovascular physiology. It maintains vascular integrity by inhibiting platelet aggregation, leukocyte adhesion and VSMC proliferation [42-46]. In addition, NO is produced in cardiac smooth muscle, where it regulates cardiac contractility [47]. Adequate levels of endothelial NO are important to preserve normal vascular physiology, and in the face of diminished NO bioavailability, there is endothelial dysfunction, leading to increased susceptibility to atherosclerotic disease [48, 49]. In many cells and for many of the biological signaling roles of NO, the physiologic target is soluble guanyl cyclase (sGC) [50]. NO activates sGC by binding to its heme moiety, resulting in increased cGMP levels. In the vasculature, cGMP mediates NO-dependent relaxation of VSMC, resulting in vasodilation [51, 52]. Another target for NO is sulfhydryl groups on proteins, to form nitrosothiol compounds [53]. Moreover, NO readily reacts with

superoxide to form the highly reactive and potentially cytotoxic anion peroxynitrite [54]. NO can also activate the enzyme poly-ADP ribose polymerase (PARP), resulting in depletion of cellular energy stores [55]. Moreover, at low concentrations (nM), NO inhibits mitochondrial respiration by reversible binding to cytochrome oxidase in competition with oxygen. At higher concentrations NO and its derivatives can irreversibly inhibit the mitochondrial respiratory chain leading to uncoupling and eventually cell death [56]. Generally, these latter mechanisms underlie some of the contrasting and toxic effects of NO, while effects on sGC and S-nitrosylation of proteins mediate many of the biological signaling roles of NO [57, 58].

1.3 The physiological NO generating systems

1.3.1 NO synthases

NO is generated in a vast number of different cell types by three different NO synthase (NOS) isoforms which catalyze a complex five electron oxidation of the amino acid L-arginine to form NO and L-citrulline. Continuous NO production is carried out by the two constitutively expressed NOSs, the endothelial (eNOS, NOS 3) and the neuronal (nNOS, NOS 1), which produce moderate amounts of NO under physiological conditions [59]. The role of eNOS is of great importance in cardiovascular and metabolic disorders since it is expressed on the vascular endothelium and is a key regulator of vascular tone and maintenance of endothelial integrity [57]. As an example, eNOS activity and expression diminishes with aging and this is linked to hypertension and many cardiovascular abnormalities [60]. Moreover, mice that are genetically ablated for eNOS are hypertensive and their resistance vessels constrict more in the presence of AngII [61]. Finally, there is a clear association between polymorphisms in the eNOS gene and type 2 diabetes [62] and reduced eNOS expression has been linked to the development of hyperinsulinemia, hyperglycemia and insulin resistance [63]. The third NOS isoform inducible (iNOS, NOS 1) was first discovered in macrophages but later found to be expressed in various cell types [64]. Upon induction, for example during inflammation iNOS produces large amounts of NO that can react with superoxide to form peroxynitrite. This iNOS-derived NO has important functions in the innate immune response. However, large quantities of peroxynitrite or chronic elevated peroxynitrite levels, can also be harmful and aid in the progression of cardiovascular disease and diabetes [65].

1.3.2 The nitrate-nitrite-NO pathway

In 1994 two independent groups reported for the first time the existence of intragastric NOS independent NO formation from inorganic nitrite [66, 67]. Moreover it was found that inorganic nitrate, derived either from the diet or from oxidation of endogenous NO, is actively concentrated by the salivary glands and excreted in saliva [68]. Nitrate is reduced to nitrite by commensal bacteria in the mouth [69]. This was the first description of the nitrate-nitrite-NO pathway in mammals and today, after 22 years of extensive research, many other fascinating aspects of this pathway have been discovered, in particular those related to systemic effects. Some of the most intriguing findings so far are that this alternative route for NO formation can improve mitochondrial efficiency and exercise performance in humans and induce vasodilation and blood pressure reduction in both healthy and hypertensive individuals [70, 71]. Some years ago research conducted in our group suggested that, apart from oral commensal bacteria, XOR can also (albeit to a lesser extent) function as a nitrate reductase [72]. The subsequent reduction of nitrite to NO has been shown to be mediated by several enzymatic or non-enzymatic factors such as XOR, deoxyhemoglobin, deoxymyoglobin, enzymes of the mitochondrial respiratory chain and also non-enzymatically

by the low pH in the stomach [73]. This alternative pathway has been shown to be of particular importance when the function of the classic NOSs, which depends on oxygen, is compromised e.g. under tissue hypoxia and conditions of oxidative stress [74]. In the past, inorganic nitrate and nitrite have been treated with some skepticism because of their proposed carcinogenic properties at high doses. However, there are still no clinical studies that clearly show causality between inorganic nitrite or nitrate and carcinogenesis [75]. On the contrary, a recent study carried out in our group found that lifelong treatment of mice with inorganic nitrate was associated with a trend towards improved survival compared to the placebo treated animals [76]. Therefore, the public opinion towards these anions has slowly started to change over time and at moderate doses, such as those achieved by vegetable consumption, they should likely be considered as beneficial substances [75].

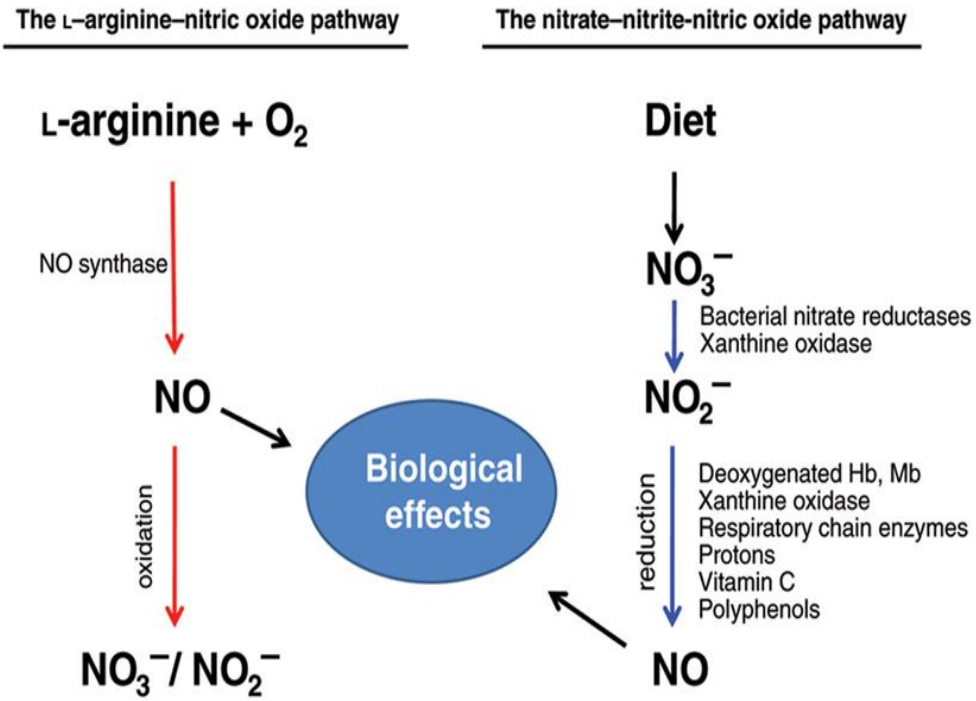

Figure 2. *Comparison of the classic NOS dependent and the alternative nitrate-nitrite-NO pathway (image from Lundberg J.O et al. 2011 [77]).*

1.4 Xanthine Oxidoreductase : a unique enzyme capable of producing both ROS and NO

XOR is a molybdoflavin enzyme that catalyzes the two terminal reactions in purine degradation, *i.e.* the oxidation of hypoxanthine to xanthine and the subsequent oxidation of xanthine to uric acid. These oxidation reactions are coupled with a parallel reduction of NAD^+ to NADH or of oxygen to superoxide and/or hydrogen peroxide [78]. The mammalian XOR is firstly synthesized in a dehydrogenase (reductase) form (XDH) but can be readily converted to the oxidase form (XO) by oxidation of sulfhydryl residues or by proteolysis. XOR is often found in dimers and each molecule of the enzyme is composed of an N-terminal 20-kDa domain containing two iron sulfur (Fe_2S_2) centers, a central 40-kDa flavin adenine dinucleotide (FAD) domain, and a C-terminal 85-kDa molybdopterin-binding (Mo-pt) domain (fig.3) [78-80].

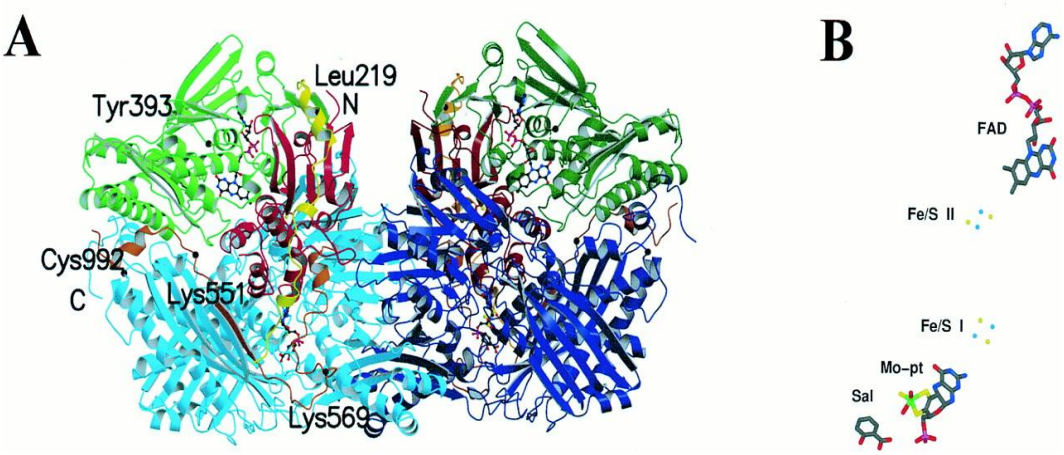

Figure 3: *(A) Molecular structure of the XOR dimer divided into the three major domains and two connecting loops. From N to C terminus, the domains are: iron/sulfur-center domain (red), FAD domain (green), and Mo-pt domain (blue). (B) Arrangement of the cofactors in one XOR subunit. The Mo ion is in green, the iron ions are in light blue, and the sulfur atoms in yellow (image from Enroth C. et al. 2000[78]).*

For many decades XOR was considered to be mostly a harmful enzyme under several pathological conditions since inflammation or ischemia-reperfusion-injuries could enhance generation of XOR-derived superoxide and hydrogen peroxide that propagates the disease process [81, 82]. Recent reports have challenged the dogma of XOR being a harmful player in many pathological conditions since it was reported that the enzyme might possess nitrate and nitrite reductase activity (fig.4) [4, 72, 83, 84]. This is interesting since it may then facilitate an alternative route of NO formation that is independent of the classical NOS dependent production. XOR is a unique enzyme in terms of being able to catalyze both the reduction of nitrate to nitrite and of nitrite to NO at the same molybdenum center [85]. The molybdenum center has to be fully reduced (Mo^{4+}) by a reducing substrate such as xanthine, hypoxanthine or $FADH_2$ in order to donate two electrons and reduce one molecule of nitrate to nitrite. Interestingly, the XOR-mediated reduction of nitrite to NO requires only one electron and therefore the fully reduced molybdenum center (Mo^{4+}) can be serially oxidized to Mo^{5+} and Mo^{6+} with the simultaneous reduction of two molecules of nitrite to NO. In the XOR-mediated full reduction of nitrate to NO, there are two consecutive/serial steps: *i)* the reduction of nitrate to nitrite, and *ii)* the reduction of nitrite to NO. In systems with high initial nitrate concentrations, the production of nitrite is much faster compared to its consumption to NO and thus a prolonged period of nitrite accumulation is observed followed by a gradual acceleration of NO production [86]. These serial reactions are highly favored under low oxygen and pH levels, but more recent findings indicate that they can actually take place, albeit to a lower extent, in conditions with normoxia and physiological pH [72, 87-90]. Although the reduction of nitrate and nitrite by XOR is kinetically rather ineffective (high km values, especially under higher oxygen tension) the final concentration of the produced NO is physiologically relevant since only picomolar amounts of bioactive NO are required for exerting any biological effects [72, 83, 86, 88, 91].

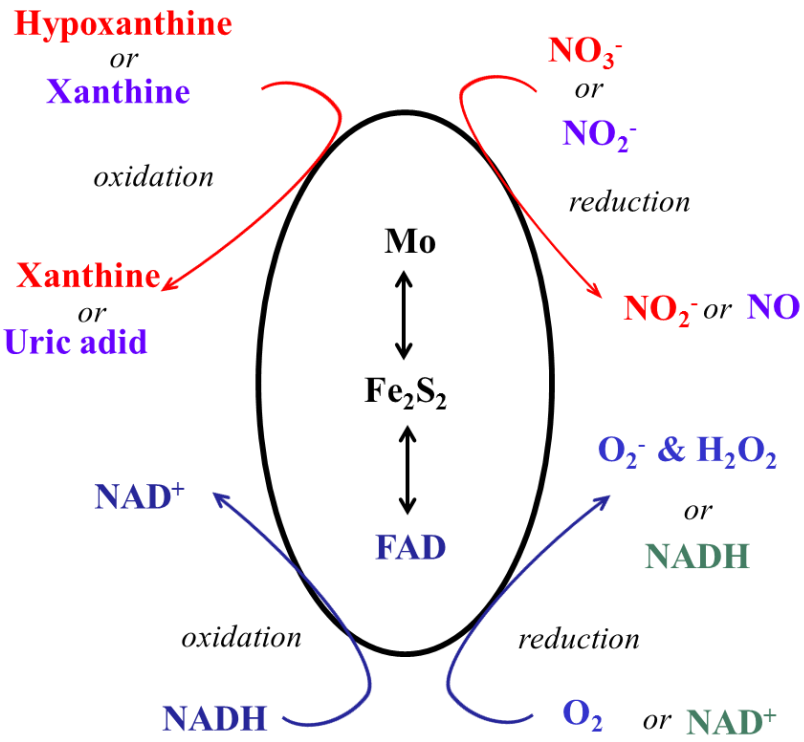

Figure 4: *Different parallel reactions that may happen in one molecule of XOR at the Mo-pt and FAD catalytic centers (modified from Boueiz et.al 2008 [92])*

1.5 The role of Adenosine type 2B (A_{2B}) receptors and AMPK as metabolic regulators in the liver

1.5.1 A_{2B} receptors and the metabolic syndrome

Adenosine is present ubiquitously and is a purine analogue and an intermediate metabolite which could lead to either formation of nucleic acids or to ATP [93]. Extracellularly adenosine acts via activation of four distinct G-protein coupled receptors denoted A_1, A_{2A}, A_{2B} and A_3 [94, 95]

The liver is one of the most important metabolic organs in the body [96]. Since the liver is the major site of glyconeogenesis, glycogenolysis, gluconeogenesis, lipolysis and lipogenesis, there is an association between abnormal liver function and the development of obesity, insulin resistance and type 2 diabetes [97].

The genetic deletion of the A_{2B} receptor from the mouse genome leads to development of metabolic syndrome and type 2 diabetes symptoms with abnormalities in the liver. In particular $A_{2B}^{-/-}$ mice on HFD have steatotic and inflamed livers with impaired insulin mediated signaling [98, 99]. In addition, $A_{2B}^{-/-}$ mice on a normocaloric diet present higher rates of liver gluconeogenesis and triglyceride biosynthesis [100]. Although the reasons why $A_{2B}^{-/-}$ mice develop such symptoms are still under investigation, one possibility is that the A_2 receptors are positively associated to the NOS dependent production of NO in various organs and cell types [101-104]. From what is mentioned above, it seems plausible that these knockout mice comprise a novel and useful model of metabolic syndrome where the efficiency of NO donating molecules e.g inorganic nitrate or nitrite could be tested.

1.5.2 AMPK as metabolic regulator

Adenosine monophosphate kinase (AMPK) is a key metabolic regulator with particular importance in maintaining liver glucose homeostasis [105, 106] and was recently suggested as a new target of the nitrate-nitrite-NO pathway [107-109]. AMPK is typically downregulated under hyperglycemic conditions and known anti-diabetic drugs such as metformin or AICAR induce AMPK activation [110, 111]. Moreover, several studies indicate that the pharmacological activation of AMPK can reduce NOX activity and expression in several target organs and cells but it still remains unknown if this inhibition on NOXs can lead to improved glucose clearance [112-115].

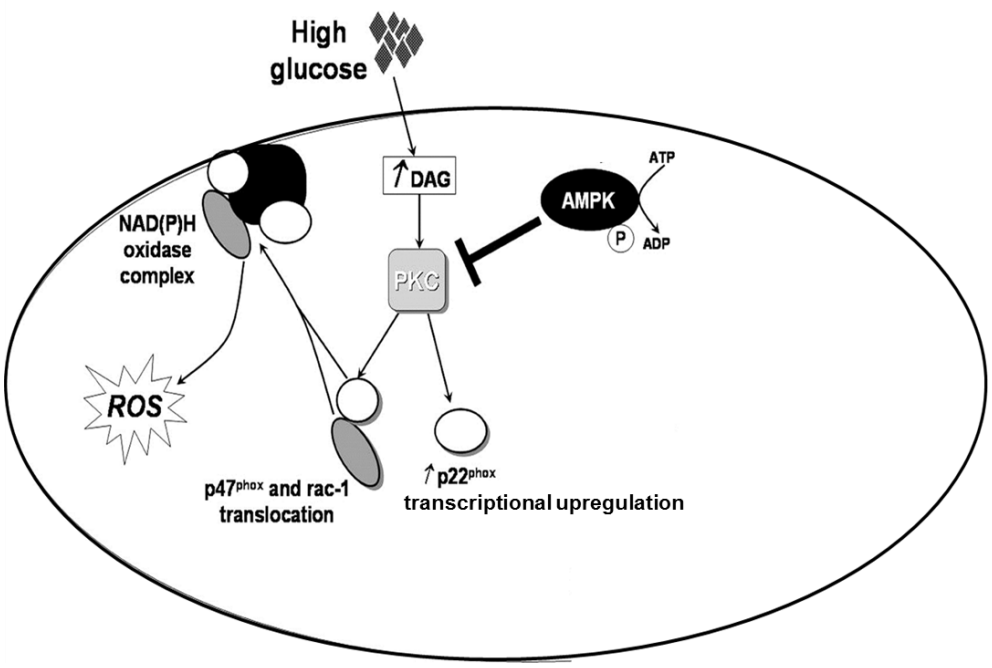

Figure 5: *Hyperglycemic conditions activate signaling pathways e.g diacylglycerol (DAG) intracellular elevation and subsequent protein kinase C (PKC) activation that create a favorable environment for NOX activation (transcriptional and post-translational level). On the contrary, post-translational modifications triggered by AMPK kinase on PKC can prevent these effects and therefore reduce the NOX-mediated oxidative stress (modified from Geolotto et al.2007 [114]).*

2 AIMS

Study I: To study if nitrate and nitrite are able to promote vasorelaxation of renal afferent arterioles and inhibit the AngII-induced contraction and NOX activity, and if this mechanism is sufficient to attenuate hypertension. Also, to clarify if the effects of nitrate and nitrite are XOR mediated.

Study II: To examine if inorganic nitrite is able to interfere with proinflammatory signals (LPS) on macrophages and monocytes. In particular, to investigate if NOX is a main target of inorganic nitrite and if the actions of nitrite are mediated via XOR-mediated reduction to NO.

Study III: To investigate if an acute dose of nitrate can improve the metabolic functions in a mouse model of metabolic syndrome ($A_{2B}^{-/-}$ mice). Moreover, to further characterize the metabolic phenotype of these mice in relation to NOX and AMPK activation.

Study IV: To investigate if nitrate can improve aging associated cardiovascular and metabolic complications in rats in relation to NOX activity and AngII receptor mediated signaling.

Study V: To further characterize the interaction between the XOR and NOS NO generating systems. To investigate if the $eNOS^{-/-}$ mice specifically upregulate and switch the function of XOR, as a compensatory mechanism to maintain NO homeostasis.

3 MATERIALS AND METHODS

Brief descriptions of the methods used in this thesis are described below. For more detailed information, please see the materials and methods sections of each scientific paper.

In vivo animal protocols

3.1 EXPERIMENTAL ANIMALS

The following strains of mice and rats were used in these studies:

Study I: male C57BL/6J mice (The Jackson Laboratory, Maine, 18-40 g), germ-free and conventional (C57BL/6J background) mice, superoxide dismutase 1 (SOD1) knockout ($SOD1^{-/-}$) and wild-type ($SOD1^{+/+}$), Sprague-Dawley rats (Charles River Laboratories, Germany).

Study III: aged (12–16months) adenosine A_{2B} receptor gene-deleted and WT mice from heterozygous breeding pairs. $A_{2B}^{-/-}$ (a gift from professor M.Sitkovsky at Northwestern University, Boston, Mass) were back crossed 11 times to a C57BL/6J background. Both sexes were used, with equal distribution for all experimental series.

Study IV: 3-month old male Sprague Dawley rats were purchased from Charles River Laboratories (Sulzfeld, Germany) and aged to 22 months in our animal facility. At the time of the experiment another batch of 3 month-old male Sprague Dawley rats were purchased from Charles River Laboratories as young controls.

Study V: age-matched (6-9 months) $eNOS^{-/-}$, $nNOS^{-/-}$, $iNOS^{-/-}$ and their respective wt mice, originating from heterozygous breeding pairs using B6.129P2-$Nos3^{tm1Unc/J}$ for $eNOS^{-/-}$, B6.129S4-$Nos1^{tm1Plh}$ for $nNOS^{-/-}$ and C57BL/6NJ.Q.$Nos2^{+/-}$ for $iNOS^{-/-}$.

All the animals (mice and rats) were housed under standard conditions of temperature (21-22ºC), humidity and illumination (12-h light/darkness). Moreover, all animals were allowed to adjust to their cage environment at least one week before the initiation of the experiments and had free access to pellet food and water. All the experiments were approved by the Regional Ethics Committees for Animal Experiments in Stockholm (Stockholms Norra Djurförsöksetiska Nämnd) and Uppsala (Uppsala Djurförsöksetiska Nämnd). All mice and rats were terminated under anesthesia with isoflurane (Forene, Abbot Scandinavia AB, Sweden), and collected organs and plasma were snap frozen in dry ice and stored at -80 ºC for later analyses.

3.2 EXPERIMENTAL PROTOCOLS-ANIMAL TREATMENTS

Study I: Telemetric devices (PA-C40, DSI, St Paul, MN) were implanted in adult male Sprague-Dawley rats. After surgery, the animals were allowed to recover for 10 days before measurements were started. Blood pressure and heart rate were measured continuously during (1) baseline conditions (72 hours), followed by giving (2) Nω-nitro-l-arginine methyl ester hydrochloride (L-NAME) in drinking water (500 mg/L) supplemented with placebo (NaCl) or sodium nitrate ($NaNO_3$, 10^{-2} mol/L) for 72 hours. The rats were then anesthetized by

spontaneous inhalation of isoflurane in air (≈2.2%), and osmotic minipumps (Alzet, Durect, CA) were implanted subcutaneously, delivering AngII (Sigma-Aldrich) at 120 ng/kg per 24 hours for 20 days. Two days after implantation, telemetric measurements were performed continuously for 15 days with ANG II infusion. During the last 4 days, measurements were conducted without placebo or nitrate supplementation.

Study III: Inorganic nitrate ($NaNO_3$; 0.1mmol/kg body weight) or placebo (NaCl, 0.1mmol/kg body weight) was administered intraperitoneally 60 min prior to the tolerance tests. A bolus of D-glucose or pyruvate was injected (2g/kg body weight; 30% in saline) and tail blood was sampled at 0, 15, 30, 60, and 120min. The mice were fasted for 6 hours (free access to water, no food) before the initiation of the experiments and separated (single caged) 1 hour prior the first series of injections. Plasma glucose was determined using a portable glucose meter (Free Style Lite, Abbot Diabetes CareInc, CA) .In a cohort of $A_{2B}^{-/-}$ mice we also investigated the effects on glucose disposal with the antidiabetic drug metformin. Metformin (0.1mmol/kg body weight) or placebo (NaCl, 0.1mmol/kg body weight) was administered intraperitoneally *(i.p)* 60 min prior to the IPGTT. In order to investigate the acute effects of nitrate in a model with more pronounced obesity, IPGTT were performed as described above in WT mice given a high fat diet-HFD (34.9%fat, D12492, Research Diets Inc., New Brunswick, NJ) for 14 months. Intraperitoneal insulin tolerance tests (IPITT) were performed similarly to IPGTT without fasting. A bolus of insulin (0.75IE/kg body weight; Novorapid 100IE/ml,Novo Nordisk A/S, Denmark) was injected (0.2IE/ml in saline) and blood samples were obtained for plasma glucose measurements.

Study IV: Initial non-invasive tail-cuff blood pressure assessments were performed. Young and old rats were divided into two treatment groups minimizing differences in initial weight and blood pressure. The groups were treated with either 10 mmol/L NaCl (placebo group) or 10 mmol/L $NaNO_3$ (nitrate) in distilled drinking water. Over treatment days 7 to 9 blood pressure was monitored again. On day 11 a glucose tolerance test was performed and tail blood samples were collected at time 0 and 30 min after glucose load for measuring insulin. The glucose tolerance test was performed as described above (study III) with the only difference being the 12 hour duration of the fasting time.

Study V: $eNOS^{-/-}$ and wt mice (6-9 months) were treated either with the selective XOR inhibitor febuxostat (Axon MedChem, 50 mg/L, initially solubilized and sonicated in a minimum volume of DMSO, 0,3 % v/v in the drinking water) or placebo (DMSO 0.3 % v/v) for 21 days. At the end of this time period, both febuxostat and placebo treated animals were additionally supplemented with 10 mmol/L $NaNO_3$ (nitrate) in their drinking water for 5 days, followed by termination similar as described above. Baseline blood pressure was monitored by non-invasive tail-cuff system in $eNOS^{-/-}$ and wt mice over 3 days before treatment initiation. The drinking water was changed every 6-7 days and the blood pressure was regularly monitored throughout the experiment until termination. Moreover, in the study aged matched $nNOS^{-/-}$, $iNOS^{-/-}$ and their respective wt mice, as well as wt mice treated with L-NAME (500 mg/L, 1 week) were used and terminated as described above for further biochemical analyses related to the function of XOR.

3.3 CELLULAR STUDIES

3.3.1 Primary cell culture of preglomerular vascular smooth muscle cells (PG-VSMC)

The preglomerular vascular smooth muscle cells (PG-VSMC) were isolated and characterized as previously described [116] with some slight modifications. In brief, the kidneys from already terminated (as described above) rats were perfused from the left cardiac ventricle with an iron oxide based solution (PBS in 1% albumin; Sigma, 1% iron oxide; Sigma). Subsequently, the kidneys were mechanically and chemically (collagenase I 1mg/ml; Sigma) treated (37ºC under shaking, 30 min) to allow dissociation of the preglomerular vessels from the surrounding tissue. The preglomerular vessels were isolated by applying magnetic force through a magnet that attracts iron oxide particles, washed 4 times with sterile PBS and then transferred in a new petri dish with appropriate medium (DMEM D0572; Sigma with 15% FBS). When the preglomerular vessels derived cells (mostly a mixture of endothelial cells and PG-VSMC) reached confluency, 3 serial trypsinization steps were performed and only the floating cells were kept for further subculture. The selected cells were characterized for PG-VSMC markers as described earlier [117] and kept up to passage 12 for further experiments.

3.3.2 Culture of mouse peritoneal macrophages (IC-21 cell line)

Peritoneal macrophages (IC-21, ATCCTIB-186; AmericanType Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, and 100 U/ml penicillin–streptomycin (Life Technologies, Grand Island, NY, USA). Cells were grown in 75-ml flasks (Sigma-Aldrich, St.Louis, MO, USA) in humidified air with 5% CO_2 at 37ºC until reaching 80% confluence. Macrophages were seeded in 6-well plates at a density of $0.5*10^6$ /ml and grown overnight in medium without FBS before starting experiments.

3.3.3 Isolation of human monocytes

Monocytes were isolated from peripheral venous blood. Whole blood from healthy donors was provided by the Karolinska Hospital Blood Bank, Stockholm, Sweden. Monocytes were separated from blood buffy coat with Ficoll under density gradient centrifugation, by using monocyte-specific anti-CD14-coatedmagnetic beads as described earlier [118]. Isolated cells were seeded with the same protocol as described above.

3.3.4 Cell treatment with pharmacological agents

The following substances were used in the experiments with the macrophages and the monocytes **(study II)**: LPS from Escherichia *coli* endotoxin (0111:B 4, 10 ng/ml), $NaNO_2$ (0.1, 10 and 1000 μM, Sigma), diethylenetriamine/NO adduct (DETA-NONOate, 0.5 mM, Sigma), Nω-nitro-L-arginine methylester hydrochloride (L-NAME, 1mM, Sigma), febuxostat (30 nM, Selleckchem), raloxifene hydrochloride (5 nM, Sigma) ,and carboxy-PTIO potassium salt (cPTIO, 50 μM, Sigma).The substances were applied into the culture medium following overnight seeding; cells were then treated for 24h.

Nitrite and DETA-NONOate were administered simultaneously with LPS, whereas all other treatments were administered 30 min prior to LPS. An additional dose of cPTIO (50 μM) was added 8 hours after the primary administration. In the case of the PG-VSMC **(study I)** the same doses of febuxostat and $NaNO_2$ were used as above. The cells were activated with AngII (Sigma, 10^{-6} M, 4 hours) and $NaNO_2$ was administrated simultaneously.

3.3.5 NADPH oxidase activity in cells

The same protocol was used in both **studies I and II**. NADPH oxidase activity was assessed by measuring NADPH oxidase derived superoxide production by lucigenin based chemiluminescence (Berthold Tubemaster, AutoLumat LB953Multi-Tube Luminometer, BadWildbad,Germany). Briefly, the cells were washed twice with PBS, harvested in 300 μl dissociation buffer and 500 μl PBS and transferred into the reaction tubes. In cases that the cells were pre-treated with a pharmacological reagent, this was added again at the same concentration. NADPH (100 μl, 100 μM) and lucigenin (100 μl, 5 μM) were added for initiating the reaction and the chemiluminescent signal was recorded every 3 sec for 3 min. For the final calculations the signal from the area under the curve (AUC) was normalized according to the cell number that each reaction tube contained.

3.3.6 mRNA extraction and qPCR in cells

The cells in all cases were harvested in RLT buffer for RNA extraction. Total RNA was isolated by using the RNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized with High Capacity cDNA Reverse transcription kit (Applied Biosystems). Quantitative PCR analysis was performed regarding to the Applied Biosystems 7500 standard protocol. Power SYBR Green Master mix (Applied Biosystems) was used for amplification and detection of DNA. PCR reaction was performed in 96-well plates with 20μl mixer/well (0.25 μmol/L of each primer and 5 μL of cDNA corresponding to the same concentration of RNA, Applied Biosystems) and in duplicates for each sample. The efficiency of PCR was calibrated according to the standard curve and the mRNA level was normalized with β-actin by the ΔCt method. Primer sequences and amplification profiles used for Nox2, Nox4, p22phox, p47phox, p67phox, XOR and β-actin are described in the tables of each individual paper.

3.3.7 NO and peroxynitrite measurements

NO (DAF-FM fluorescence)

The cells were plated in a 96-wellplate and DAF-FM solution was added (Sigma, 10 μM, 45 min). Cells were then washed and harvested in PBS containing L-arginine (1.15mM) with or without L-NAME (1mM). The cells were then transferred to a black 96-well microplate (Nunc, ThermoScientific) and DAF-FM was excited with light at 495nm from a fluorometer (Spectra Max Gemini, Molecular Devices Corporation, Downington, PA, USA) and the emission at 515nm was detected (37 ºC). For the final calculations the change of the DAF-FM derived fluorescence from the treated cells was expressed as the percentage change from the respective control ones.

Peroxynitrite (HPF fluorescence)

Hydroxyphenyl fluorescein (HPF, Sigma Aldrich) derived fluorescence was used for the measurement of the highly reactive oxygen species, hydroxyl radical and peroxynitrite according to the manufacture's protocol. Uric acid (1mM) was then used to scavenge peroxynitrite.
The fluorescence signal was measured at an excitation wavelength of 490 nm and an emission wavelength of 515nm (37 ºC). Peroxynitrite production was calculated by subtracting the HPF signal from the HPF+uric acid signal. The result was expressed as relative changes compared to control.

3.3.8 Cell viability measurement

The cell viability was assessed with the Trypan Blue exclusion test (0.4% , Gibco®) where the cells are incubated for 5 min with the trypan blue dye for 5 min (37 ºC) and then are counted with the help of a hematocytometer. The alive cells do not uptake the dye and remain unstained while the dead ones are stained blue.

3.4 ANIMAL-TISSUE AND ENZYME STUDIES

3.4.1 NADPH oxidase activity in kidney cortex, liver and heart homogenates

The NADPH oxidase activity was measured as described at the section **3.3.5** with the only difference that tissue homogenates of similar concentrations were used instead of cells. In all cases the final chemiluminescent signal was normalized according to protein concentration (protein quantification with the Bradford assay according to manufacturer's instructions, Protein Assay Dye Reagent Concentrate; Bio-Rad). The kidney cortex and liver tissues were homogenized in the Bullet Blender (Next Advance, Inc.) with zirconium oxide beads 0.5 mm, centrifuged (2000 g, 20 min, 4 ºC) and the supernatant was properly diluted and snap frozen in dry ice and kept in -80 ºC until the day of analysis. In the heart the protocol was slightly modified as following: a slice from the left ventricular area was homogenized in a buffer (Tris-HCl, pH 8.0, 1 mmol/L, NaCl 150 mmol/L, and EDTA 5 mmol/L) with stainless steel beads in the bullet blender. The supernatant was collected after centrifugation at 4°C for 20 min at 12000 g, diluted 1:5 in PBS and 5 μmol/L lucigenin was added. Finally, 300 μmol/L NADPH was added and the NADPH oxidase activity was determined.

3.4.2 Xanthine oxidoreductase activity (O_2^-, H_2O_2, NO) assays in liver homogenates and with purified enzyme

XOR mediated superoxide production

The principal of this assay is very similar to the above described (NADPH oxidase activity) with the difference that xanthine is used as a substrate instead of NADPH. Moreover, and because of the technical obstacles in getting a reliable XOR derived signal, the homogenization protocol was modified in the following ways: pieces of livers were homogenized in the bullet blender as above and in 2 volumes of a buffer (Tris-HCl, pH 8.0, 1 mmol/L, NaCl 150 mmol/L, and EDTA 5 mmol/L) that contained protease inhibitors (1/1000 v/v dilution). The homogenates were centrifuged at 12000 g for 20 min at 4°C and the pellet was collected and resuspended in a final volume of 1 ml PBS that contained 50 μM lucigenin (Sigma) and 100 μM xanthine (Sigma). Superoxide levels were determined by measuring lucigenin chemiluminescence every 3 s for 1.5 min at 37 °C with the AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized

to the protein concentration (Protein Assay Dye Reagent Concentrate; Bio-Rad). XOR-derived superoxide production was also measured in a cell-free system using purified XOR (0.001 U/ml, Roche), 50 μM lucigenin and 10 μM xanthine in a total volume of 1 ml PBS. As described above, superoxide generation was measured by chemiluminescence for 60 s with the AutoLumat LB953 Multi-Tube Luminometer with and without sodium nitrite pretreatment (1 mM, 30 min).

XOR mediated H_2O_2 production

The Amplex Red derived fluorescence was used in order to measure the XOR mediated H_2O_2 production. In brief, liver pieces were homogenized in a lysis solution (sucrose 250 mM, Tris-HCl 10 mM, pH 7.4) in the bullet blender. After centrifugation (500 g, 10 min, 4 ºC) the liver supernatant was diluted in Tris-HCl 100 mM (pH 7.5) in a final concentration of 1.5 μg/μl and the homogenate was added in a 96-well microplate (fluronunc black, Nunc) and incubated with or without febuxostat (1μM, 30 min, 37ºC in dark). Each reaction contained 80 μM Amplex Red (Molecular Probes, Life Technologies, Stockholm, Sweden,) reagent, 0.8 U/mL HRP (Sigma) and 16 μM hypoxanthine (Sigma) in 100 mM Tris-HCl, pH 7.5. After 30 minutes incubation in dark (37ºC), the fluorescence was measured for 120 min in a fluorescence microplate reader using excitation at 530 nm and fluorescence detection at 590 nm. The signal was normalized according to the protein concentration (Protein Assay Dye Reagent Concentrate; Bio-Rad) and presented as % changes of the respective wt animals. The same assay was used for measuring the H_2O_2 production from purified XOR (Roche, 5-0.08 mU/ml) directly diluted into the reaction buffer containing Amplex Red as previously described. Finally, the direct effects of inorganic nitrite on the XOR-derived H2O2 were tested after pre-incubating the enzyme (1mU/ml) with sodium nitrite (1 mM, 30 min) and increasing doses of catalase (1-1000 U/ml, Sigma).

XOR mediated nitrite to NO reduction (NO analyzer)

Liver homogenates (100 mg/ml, homogenized as described in the **3.4.1** section) were added into a chamber of an Oxygraph-2k system (Oroboros instruments) under nitrogen atmosphere (oxygen ≤ 0.02 %) at 37 °C. The oxygraph chamber was connected in line to a NO analyzer device (ECO Physics analyzer, CLD 77 AM, Swiss), using nitrogen gas as carrier (400 mL/min). After a period of equilibration (approx. 20 min), $NaNO_2$ (1 mM) was injected into chamber and the NO production was recorded real-time throughout the experiments, using a data acquisition system (AcqKnowledge v3.9, Biopac MP150). The NO production was recorded during 1 h after nitrite injection and, in a subset of experiments, the selective XOR inhibitor febuxostat (100 nM) was added 15 min before $NaNO_2$ addition. The average NO signal (ppb) was calculated in a 5 min period during the baseline and the maximum response to nitrite. Nitrite-mediated NO release was calculated by subtracting the average NO values after the addition of $NaNO_2$ from the NO values at baseline. The involvement of XOR was assessed by statistically comparing the tissue homogenates response with or without febuxostat which is a highly selective XOR inhibitor. The same assay was performed by using purified XOR (Roche, 0.05 U/ml) in the presence of xanthine (10 μM) with or without $NaNO_2$ (1 mM).

3.4.3 mRNA extraction and qPCR in kidney cortex, heart, mesenteric arteries, liver and aorta

The mRNA extraction and qPCR protocols were the same as those performed for the cells with the difference that homogenization buffers and protocols were used were modified as described:

Kidney cortex: The kidney was homogenized in a proper volume of RLT buffer and in the bullet blender with RNase free zirconium oxide beads (0.5 mm). The mRNA was extracted according to the instructions of the RNeasy kit (QIAGEN, Sollentuna, Sweden).

Heart: Heart tissue from the left ventricular area was homogenized in a proper volume of Trizol (Life Technologies) in the bullet blender with RNase free stainless steel beads. The mRNA extraction was a combined protocol from the Trizol's manufacturer instructions and the use of columns from the RNeasy kit (QIAGEN, Sollentuna, Sweden) that led to better mRNA yield and purity.

Mesenteric arteries: Isolated mesenteric arteries (3-8 mg) were homogenized in 1 ml Trizol and RNase free stainless steel beads. The mRNA was extracted with the same protocol as for the heart.

Liver: The same protocol as for the kidney cortex was used.

Aorta: The same protocol as for the mesenteric arteries was used.

3.4.4 Western blot in liver and aorta

Both liver and aorta were homogenized using 0.5 mm zirconium oxide beads by using the bullet blender and in lysis buffer containing 10mM Tris-HCl (pH 8), 150mM NaCl, 5mM EDTA, 60mM N-octylglucoside,1% TritonX-100, protease, and phosphatase inhibitor cocktails (Sigma-Aldrich, Stockholm, Sweden). After centrifugation and protein quantification of the soluble fraction (Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Solna, Sweden), equal amounts of protein were separated by SDS-PAGE followed by transfer to a PVDF membrane (Bio-Rad).The membranes were blocked with 5% non-fat dry milk in Tween-containing TBS (TBS-T), incubated with specific primary antibodies as indicated on each study. After overnight incubation with the primary antibody, the membranes were washed with TBS-T and incubated with the appropriate secondary antibody and the Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad). The bands' intensities were quantified using densitometry (Image Lab 5.2.1 software, Bio-Rad) and results are reported as relative optical density of the specific proteins.

3.4.5 Plasma markers

Nitrate and nitrite

Nitrate and nitrite were measured by HPLC (ENO-20) and autosampler (840, EiCom, Kyoto, Japan). In brief, the plasma samples were extracted using methanol (1:2) and then centrifuged for 10 min 4°C 10000 g. Nitrate and nitrite were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm.

cGMP

cGMP was measured in plasma samples containing IBMX (10 μM) for preventing cGMP degradation with ELISA (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions.

Insulin

Insulin was measured in plasma by using the proper ELISA kit (Mercodia, Uppsala Sweden). Based on the insulin and respective glucose values, the HOMA-IR score was calculated under fasting and after glucose treatment for study III and under fasting for study IV.

Arginine and Citrulline

25 µl of plasma were crashed with 225 µl of 0.2 % formic acid in isopropanol containing the internal standard (0.73 µmol/L of N_4-Arginine). After centrifugation (8000g, 10 min) the supernatants were collected and analyzed by liquid chromatography tandem mass spectrometry (LCMS/MS). Separation was performed on an ACQUITY UPLC System from Waters Corporation (Milford, MA, USA) using an Atlantis HILIC Silica 3 µm (150 X 2.1 mm) column from Waters. Mobile phases consisted of 0.2 % formic acid in ACN:MeOH (75:25) and 0.2 % formic acid in water. The flow rate was set at 400 µl/min. Detection was performed using a Waters Xevo® TQ triple quadrupole equipped with an Electrospray Ion Source working in positive mode. The selected reaction transitions monitored were 175.1/70.1, 176.1/70.1 and 179.1/71.0 for arginine, citrulline and N_4-arginine, respectively. Arginine and citrulline were chromatographically resolved at the baseline.

Creatinine

Levels in plasma, as a marker of renal function, were analyzed according to the manufacturer's protocol, using the creatinine assay from Cayman Chemical (Ann Arbor, MI, USA).

Renin

EDTA-plasma was incubated with a surplus of substrate-enriched plasma from a nephrectomized sheep for 24 h. The plasma renin concentration was measured by the rate of AngI formation, and AngI was detected by a radioimmunoassay through the antibody trapping method of Poulsen and Jørgensen [119] with minor modifications. The assay was standardized in terms of international units of renin per liter by the activity of the World Health Organization International Renin Standard (Ref. 68-356, National Institute for Biological Standards and Control, Hertfordshire, UK). Samples of 0.05 IU/l were included in every run of the renin assay. In the period of measurement, 1 IU of the World Health Organization standard corresponded to 4.3 0.8 ng AngI/h. The between-assay coefficient of variation was 19%.

AngII

Plasma samples were extracted according to manufacturer (Sep-Pak Classic C18 cartridge, WAT051910) and the dried eluates were stored at -80°C until the time of the assay. The Plasma samples were incubated with a specific anti-AngII antibody (Ab-5–030682) for 24 h and with tracer 125I-labeled AngII (kindly provided by the Dept. of Clinical Physiology, Glostrup Hospital, Denmark) for another 24 h. Free antigen was removed by adding a charcoal-plasma suspension, and, after centrifugation, radioactivity of the supernatant was measured. The detection limit was 1-2 pg/ml. Values were corrected for the extraction recovery of unlabeled AngII (~75% in the present analyses) added to plasma in the individual assay. Intra- and inter-assay coefficients of variation were 5 and 11 %, respectively.

Uric acid

Plasma samples were incubated in black microplates (fluronunc black, Nunc) for 30 min at 37 °C, protected from light, together with a working solution of 80 μM Amplex Ultra Red reagent (Molecular Probes, Life Technologies, Stockholm, Sweden) containing 0.8 U/ml horse radish peroxidase (Sigma, Cat.no: P-8250) and 2 U/ml uricase (Sigma), in 100 mM Tris-HCl pH 7.5. A standard curve (50 μM to 195 nM uric acid) was prepared and run at the same time. The fluorescence was measured in a microplate reader (Molecular Devices, excitation 530 nm and emission 590 nm) and the concentration of uric acid was calculated according to the standard curve. The same plasma samples were also analyzed for uricase activity, which did not differ among the experimental groups (data not shown).

3.4.6 NO measurement (DAF-FM fluorescence) in afferent arterioles and liver

The method relies on the same principle as described on the section **3.3.7** with some slight modifications that make it more suitable for measurements in afferent arterioles and liver respectively. Regarding the afferent arterioles, DAF-FM was loaded into afferent arterioles by adding the esterified form to the bath (2x10-5 mol/L, 45 min, room temperature). DAF-FM was excited with light at 480nm from a Thiel monochromator using a Nikon eclipse, TE300 microscope (Thiel, Munich, Germany, Nikon Stockholm, Sweden), and the emission isolated at 535nm detected with back-illuminated EMCCD camera (DU-887, Andor Technology, Belfast, Northern Ireland) under software control by MetaFluor (Molecular Devices Corporation, Downington, PA, USA). Digital pictures were acquired every 30 seconds at a magnification of x1300. Change in fluorescence intensity of DAF-FM with sodium nitrite (10-5 mol/L), as an indicator for NO production was quantified as the % change from the initial value. Liver homogenates in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4 (7 mg/ml protein) were incubated with 500 μM $NAD^{+,}$ 500 μM NADH and 1 mM NADPH using PBS as a buffer. Following the addition of DAF-FM (10 μM), 150 μl of homogenates were transferred in duplicates to black microplates (FluoroNunc black, VWR, Stockholm, Sweden) and the fluorescence was measured in a microplate reader (Molecular Devices, Wokingham, Berkshire, UK) using excitation 495 nm and emission 515 nm. Fluorescence was measured every 60 min for 10 hours at 37 °C. For the final analysis the average fluorescence/hour was calculated for each animal.

3.4.7 Superoxide production (DHE staining) in aortic rings

Fresh segments of thoracic aorta from each rat were snap frozen in optimal cutting temperature compound (OCT). Cryosections (10 μm) were incubated in the dark with DHE (Sigma, 2 μmol/L) for 30 min at 37°C. Images were captured immediately using a Zeiss Axiophot fluorescence microscope at excitation and emission wavelengths of 520 and 610 nm. For each aortic ring, DHE fluorescence was measured throughout the total vessel wall and calculated from four separate high power fields from each quadrant of the ring. DHE fluorescence was quantified using image J software (NIH, Bethesda, MD, USA).

Ex vivo vascular reactivity studies

3.4.8 Vascular reactivity of renal afferent arterioles

Dulbecco's modified Eagle's medium (DMEM/F12) with 0.01 mol/L HEPES (Invitrogen AB, Lidingö, Sweden) was used for dissection, bath, and perfusion. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA:SERVA Electrophoresis Heidelberg, Germany). The concentration of BSA was 0.1% in dissection and bath solutions, and 1% in the perfusion solution. Dissection of renal cortical afferent arterioles was performed at 4°C. Single isolated arterioles with their glomeruli were perfused in a thermo-regulated chamber (37°C) using a micromanipulator perfusion system (Vestavia Scientific, Vestavia Hills, AL, USA), which allowed adjustment of outer holding and inner perfusion pipettes. The chamber and the perfusion system were fixed to the stage of an inverted microscope (Nikon, Badhoevedorp, Netherlands). The perfusion pipette, with a diameter of the tip of 5-μm, was connected to a reservoir containing the perfusion solution. The criteria for the use of an arteriole were: an intact myogenic response and a satisfactory, remaining basal tone. Increasing perfusion pressure rapidly and assessing the change in the luminal diameter, which produced a constriction, tested both criteria. A further criterion was a fast and complete constriction in response to KCl (100 mmol/l) solution. The experiments were digitally recorded and then digitized off-line and analyzed as described before [120]. Changes in luminal diameters were measured to estimate the effect of vasoactive substances. The equipment used allowed a resolution of 0.2 μm of the vessel structures. In all series, the last 10 seconds of a control or treatment period were used for statistical analysis of steady state responses. Each experiment used a separate dissected afferent arteriole.

3.4.9 Wire myography in carotid, interlobar and mesenteric arteries

The above mentioned arteries were isolated from C57BL6 mice (**study I**) or mesenteric arteries only from Sprague-Dawley rats (**study IV**) were isolated and dissected in ice-cold Krebs solution. Arterial rings (2mm) were mounted on 40μm stainless steel wires (carotid artery) or 25μm tungsten wires (interlobar and mesenteric arteries) in a small vessel myograph (Model 620M, Danish Myo Technology, Denmark) for recording isometric force by transducers (PowerLab 4/30, AD Instruments, Australia). The chambers were filled with Krebs solution (37 °C, pH 7.4) aerated with carbogen (95% O_2, 5% CO_2). Resting tension of arteries was set according to the normalization procedure [121] and vessel viability was assessed by the responses to 0.1 mol/L KCl. Contractile responses were expressed as percentage of constriction to 0.1 mol/L KCl (% of KCl).

4 RESULTS AND DISCUSSION

4.1 NADPH oxidase is a major target and possible regulator of the nitrate-nitrite-NO pathway (studies I, II, III and IV)

4.1.1 NOX in the renal microvasculature is a primary target for the blood pressure lowering effect of inorganic nitrate in AngII-mediated hypertension (study I)

The activation of the renin-AngII system (RAS) is an important physiological mechanism for maintaining blood pressure homeostasis. After binding to the AT_1 receptor on the cell membrane AngII promotes intracellular formation of superoxide by upregulating and activating NOX [122]. These oxidative stress conditions contribute significantly to enhanced arteriolar reactivity in the renal microvasculature and susceptibility to hypertension [120, 123]. The renal afferent arterioles are the major resistance vessels in the kidney and play a critical role in blood pressure regulation [124, 125]. In contrast to the effects of superoxide, compounds that act as NO donors can attenuate the AngII-induced contractile responses in isolated perfused renal afferent arterioles [126]. Therefore, the balance between superoxide and NO is of importance for determining the reactivity of renal afferent arterioles and pharmacological interventions that lower the levels of the former and increase the latter could be of significant interest [127].

Earlier studies had indicated that dietary nitrate and nitrite have blood pressure lowering effects in normotensive and hypertensive individuals [128-131]. Moreover, animal studies by Carlström et al. [132] and Montenegro et al. [133] had indicated that dietary nitrate and nitrite attenuate oxidative stress in the cardiovascular system and may downregulate vascular NOX. However, the details of the mechanism and the particular importance of oxidative stress in the renal microvasculature remain unclear.

In **study I** we performed several experiments on isolated renal afferent arterioles, preglomerular vascular smooth muscle cells (PG-VSMC) and whole animals in order to test the hypothesis that stimulation of the nitrate-nitrite-NO pathway targets NOX in this particular vascular bed. As shown in fig.3C (**study I**) nitrite attenuates renal microvascular responses to AngII to a higher extent compared to that observed during NOX inhibition with apocynin. In order to further confirm the direct effects of nitrite on the NOX, PG-VSMC and renal cortex (which is rich in preglomerular vessels) were isolated and their NOX activity was measured after proper incubation (30 min) with AngII and/or nitrite. As shown in fig.4A-D from **study I**, in both cases AngII strongly elevates NOX activity, which is normalized in the presence of nitrite. Moreover, nitrite alone has a slight inhibitory effect on NOX compared to untreated controls but not as strong as in the presence of AngII. Overall, our results indicate that the effect of nitrite is stronger in conditions where NOX activity is enhanced. In agreement with that, when the renal afferent arterioles are pretreated with apocynin, nitrite cannot not have any further additive vasodilatory effect (fig 3A-B. **study I**).

Although, from **study I** we obtained convincing evidence that NOX comprise a major target of the nitrate-nitrite-NO pathway there were some questions that warranted further investigation and were addressed more in detail in the following studies. In particular, we identified four possibilities regarding the exact mechanism by which nitrate and/or nitrite can lower NOX activity: a) direct scavenging of superoxide by NO, b) reduction in the number of NOX subunits at the mRNA or protein level, c) post-translational modifications on the NOX subunits e.g nitrosylation of critical thiols in the catalytic site of the enzyme or nitration reactions, d) change in other signaling proteins that mediate post-translational NOX

activation. The mRNA levels of the different NOX subunits did not change in the presence of nitrate (fig.S4 in **study I**) and therefore this possibility is unlikely although more detailed time-course studies would be warranted in order to better answer this question.

Another important issue in relation to the findings from **study I** that requires further investigation is the relevant contribution of the NOX inhibition and the cGMP dependent pathways in the nitrite mediated vasodilation. The NOS-derived NO signals mainly through the sGC and cGMP and sGC activation mediates many of the beneficial effects of endogenously produced NO in the cardiovascular system [134]. Other small signaling molecules e.g H_2S are also able to increase NO biovailability and cGMP levels but compared to the NOS-derived NO are more promiscuous and affect multiple signaling pathways [135]. Similarly, in **study I** we show that the vasodilatory effects of nitrite are partially mediated by sGC (fig.2C-D) whereas in another study we observed that nitrite acts in a cGMP independent manner in renal interlobular arteries subjected to hypoxia [90]. NOX-derived superoxide induces eNOS uncoupling, reacts with NO and forms peroxynitrite and can also directly oxidize/deactivate sGC [136]. Therefore, NOX inhibition is a promising approach for restoring the eNOS-NO-sGC-cGMP mediated signaling. It remains to be elucidated if NOX inhibition is the primary outcome of the nitrate-nitrite-NO pathway which in turn facilitates, to some extent, cGMP-mediated signaling or if these are two rather independent events.

4.1.2 Inorganic nitrite reduces the NOX mediated superoxide production in activated macrophages via a NO dependent mechanism (study II)

Inflammation is a hallmark for the progression of most of the cardiovascular and metabolic disorders and activated macrophages with elevated superoxide production can aggravate this pro-inflammatory phenotype [137-140]. We had previously shown that nitrate and nitrite possess anti-inflammatory actions [141, 142] but it remained unclear if this pathway could have direct effects on immune cells. Since our results from **study I** depicted NOX in the renal microvasculature as one of the major targets of the nitrate-nitrite-NO pathway, in **study II** we wanted to further expand these findings and test if nitrite exerts effects on activated macrophages via a similar mechanism. As shown in fig.1A (**study II**) macrophages stimulated with LPS showed significantly higher NOX activity compared to control cells which was normalized back to baseline levels after nitrite co-administration. However, when the cells were pretreated with the NO scavenger cPTIO, NOX mediated superoxide production was significantly elevated and nitrite was unable to reduce the LPS induced NOX activity (fig.2A, **study II**). As shown in fig.5A of **study II** and similarly to **study I**, the mRNA levels for Nox2, which is the most abundantly expressed NOX subunit were not changed after treatment with nitrite. Moreover, the gene expression of the other NOX subunits did not change in response to nitrite in the activated macrophages (*data not shown*). Finally, LPS treatment elevated the levels of peroxynitrite (fig.5C in **study II**) in macrophages but this effect was attenuated in the presence of nitrite indicating that a direct scavenging of superoxide by nitrite-derived NO and the formation of peroxynitrite is not a very likely mechanism. In conclusion, nitrite attenuates NOX activity of LPS activated macrophages via a mechanism that involves NO production. This attenuation does not seem to derive from downregulation at the mRNA level or from direct scavenging of superoxide. Therefore, nitrite derived NO more likely acts at the translational and post-translational level either directly on NOX or on important signaling proteins that regulate its function. Interestingly, nitrite alone is not able to downregulate NOX activity or increase NO production in unstimulated macrophages, indicating (as in **study I**) that stimuli which act as strong NOX activators create a favorable environment for a more potent action of nitrite.

4.1.3 Inorganic nitrate targets liver NOX activity in aged and metabolically dysregulated $A_{2B}^{-/-}$ mice: Possible links with AMPK activation (study III)

In **study III**, we focused on a mouse model of metabolic syndrome ($A_{2B}^{-/-}$ mice) which is characterized by obesity, hyperglycemia, worsened glucose clearance, hepatic inflammation and impaired IRS-2 mediated signaling in the liver [99]. Apart from our previous focus on NOXs, we wanted to investigate if inorganic nitrate is able to alter the phosphorylation/activation levels of AMPK which is an important regulator of glucose homeostasis in the liver and closely linked to NOX activity [105, 106]. Many studies suggest that elevated AMPK activity downregulates NOX activity. For example, signaling pathways triggered by drugs that act as AMPK activators can upregulate the expression of antioxidant enzymes e.g SOD2 or prevent the translocation of the p47phox subunit from the cytoplasm to the cell membrane and therefore lead to lower NOX mediated superoxide production [112, 143].

However, it has also been shown that activation of NADPH oxidase activity and in particular NOX2 is associated with AMPK inhibition in colon cancer cells [144]. In addition, it is known that elevated liver NOX activity can diminish glucose uptake and contribute to the development of hyperglycemia [37]. Conversely, antidiabetic drugs such as metformin or AICAR that lead to higher AMPK activation can improve glucose uptake and metabolism in the liver [111]. Therefore, pharmacological interventions that target both NOXs and AMPK might be promising in treating metabolic abnormalities.

As shown in fig.5 and 6 of **study III,** $A_{2B}^{-/-}$ mice are characterized by elevated NOX activity and impaired AMPK phosphorylation in the liver. Interestingly, *i.p* administration of metformin or inorganic nitrate was able to significantly reverse these phenomena and improve glucose clearance (figure 2 in the article). Moreover, and in agreement with our two previous studies, inorganic nitrate had no effect in wt mice which have a lower NOX activity compared to the $A_{2B}^{-/-}$. Another subsequent study also suggested that inorganic nitrite resembles the action of metformin and can enhance glucose uptake in skeletal muscle via AMPK activation [109].

In conclusion, our results from **study III** indicate the following possibilities: a) the nitrate-nitrite-NO pathway first decreases NOX activity and then this attenuation facilitates AMPK activation, b) the nitrate-nitrite-NO pathway first activates AMPK which in turn triggers signaling pathways that lower NOX activity or c) a and b happen and reinforce each other simultaneously. Since, the activation of AMPK with metformin led to a significant reduction in NOX activity and this has been confirmed also in previous studies, the possibility that nitrite acts in a similar way is plausible. However, despite similarities in the observed effects, nitrate and metformin are two vastly different molecules that also have distinct mechanisms when it comes to glucose clearance [145, 146]. Therefore, in order to fully depict the series of events in our experimental setting future studies with the simultaneous use of nitrate and AMPK or NOX inhibitors are required.

4.1.4 NOX activity and Ang II-mediated receptor signaling are downregulated by inorganic nitrate in aged and hypertensive rats (study IV)

Natural aging is associated with accumulation of visceral fat and increased risk for cardiovascular complications [147, 148]. Several molecular mechanisms contribute to the development of aging associated cardiac and metabolic disorders. Among them endothelial dysfunction with reduced eNOS dependent NO production and dysregulation of the RAS system have been proposed to play a role [149, 150]. AngII acts on two different receptor types called AT_1 and AT_2. Activation of AT_1has been linked to higher NOX mediated ROS production, induction of vasoconstriction and therefore possible detrimental effects in the cardiovascular system, whereas the opposite function has been suggested for the AT_2 receptor [151]. Moreover, elevated NOX activity can lead to transcriptional upregulation of the AT_1 receptor whereby a vicious cycle is created [152]. Aging is often characterized by an imbalanced higher expression ratio of the AT_1/AT_2 receptors, higher NOX activity and elevated AngII mediated signaling [153]. Considering that we already showed in **studies I-III** that NOXs are a major target of inorganic nitrate and nitrite, in **study IV** we wanted to further investigate the therapeutic potential of a short term treatment with sodium nitrate in a natural model of aging.

As shown in fig. 4-6 of **study IV,** aged rats were characterized by increased NOX mediated superoxide production (fig.4), AT_1/AT_2 ratio (fig.6) in different parts of their cardiovascular system, and enhanced AngII-mediated contractions in mesenteric arteries that were blocked by a NOX inhibitor (fig.5). Interestingly all these parameters were almost normalized to the level of young animals after two weeks administration with inorganic nitrate in their drinking water. This was associated *in vivo* with a significant reduction of MAP (~12mm Hg) and an improvement of the metabolic function without altering renin or AngII expression levels (figures 1, 2 and 6J-L, **study IV**). Although, we were not able to fully depict the series of events our proposed mechanism suggests that nitrate is converted to nitrite and then to NO which most likely lowers NOX activity. This is similar to our results from **studies I-III**. This reduction of ROS could lead to a lower transcriptional activation of the AT_1 receptors and contribute to the observed lower contractions to AngII in the resistance vessels originating from the nitrate treated old animals. These results are in agreement with studies where classical NO donors were used and downregulation of AT_1 receptor mRNA expression occurred whereas NOS inhibition resulted in the opposite effect [154-156]. Moreover, other studies have indicated that molecules that lower ROS and in particular superoxide e.g tempol or apocynin can also normalize the transcriptional levels of the AT_1 receptors [157, 158].

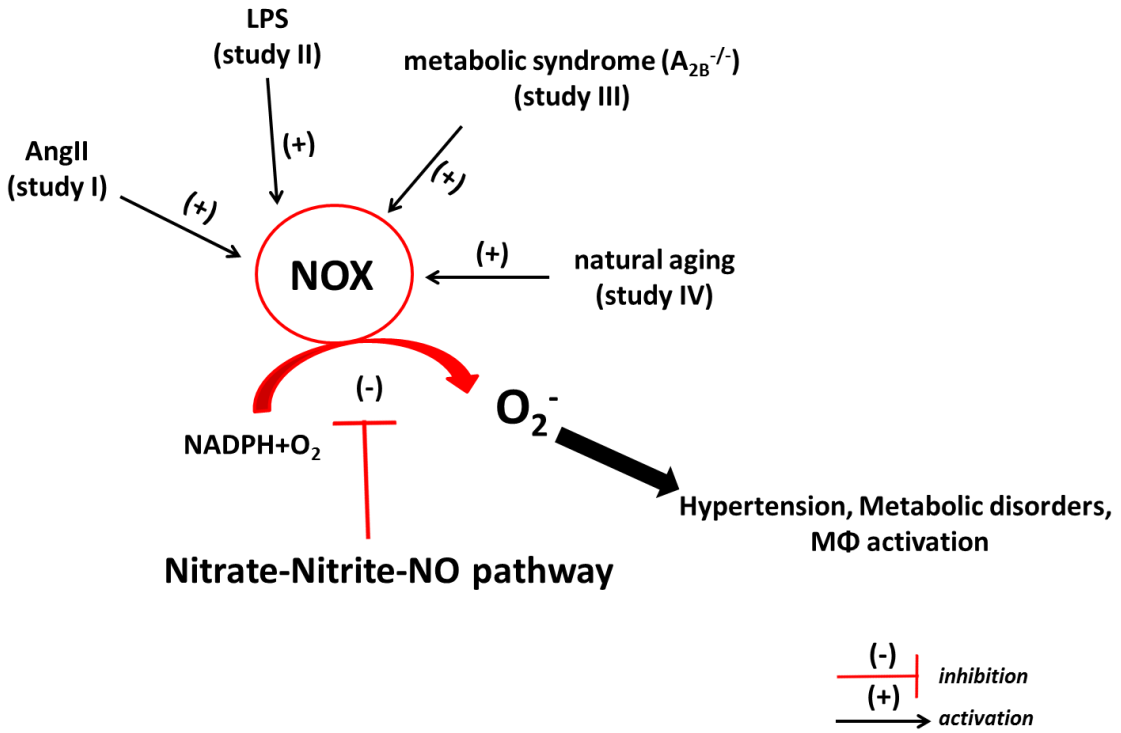

Figure 6: *Summarized overview of the studies I-IV indicating that NOX is a major target of the nitrate-nitrite-NO pathway (MΦ: macrophages).*

4.2 The relation between the nitrate/nitrite and NOS mediated NO production (studies I, II, IV and V)

Several studies carried out by our group and others have indicated that the nitrate and/or nitrite-mediated NO production happens in a NOS independent manner and it can e.g reverse features of the metabolic syndrome or be cardioprotective in the $eNOS^{-/-}$ mice [159-161]. Despite these findings, the exact interplay between these two different NO generating systems remains unclear. In the current thesis we attempted to further address this question, in particular in **study V**. Our overall conclusion is that the nitrate-nitrite-NO pathway not only acts independently of the NO synthases, but it is actually potentiated during compromised eNOS function.

4.2.1 The vasodilatory effect of nitrite is still present and potentiated upon NOS inhibition (study I)

In particular, in **study I** we observed that the maximum nitrite mediated dilatation (% change of the arteriolar diameter) was about 5% in untreated renal afferent arterioles. However, if the vessels were pretreated with the NOS inhibitor L-NAME this dilatation was increased up to ~15% (fig.1A & C, **study I**). Our results also revealed that nitrite alone attenuates the AngII-mediated maximum contractility by ~40% but if the same experiment is repeated in the presence of L-NAME the effect of nitrite is at least twice as strong (fig.2A, B and D, **study I**). In a previous study from our group, a crosstalk between eNOS and nitrate mediated NO production in rodents with functional NO synthases in their vasculature was found [162]. The limitation of both of these studies is that L-NAME which is a general NOS inhibitor was used. However, it remained unclear if the situation is the same in mice where eNOS is specifically genetically ablated and this was addressed in **study V**.

4.2.2 The nitrate-nitrite-NO pathway is highly potentiated in eNOS$^{-/-}$ mice (study V)

Previous studies in disease models such as myocardial IRI, intimal hyperplasia, diabetes etc had suggested that the beneficial effects of the nitrate-nitrite-NO pathway are not blocked under pharmacological inhibition of NOSs or in the eNOS$^{-/-}$ mice [163-166]. However, most of these studies had focused on hypoxic conditions and did not assess in detail the interrelation between the two pathways. In **study V** we used wt and eNOS$^{-/-}$ mice treated with a high dose of nitrate (10 mM) in their drinking water for 5 days. In agreement with our crosstalk hypothesis, the blood pressure was paradoxically elevated by nitrate treatment in wt mice whereas there was a significant lowering of blood pressure in the eNOS$^{-/-}$ animals (fig. 6D, E & F, **study V**). Moreover, eNOS$^{-/-}$ mice were characterized by a more efficient nitrate to nitrite conversion 1 hour after *i.p* administration with inorganic nitrate (fig.2, **study V**). Finally, livers from the eNOS$^{-/-}$ mice generated much more XOR-derived NO from both nitrate and nitrite as measured by DAF-FM fluorescence and actual NO gas formation (fig.3, **study V**) confirming our hypothesis of a more efficient nitrate to nitrite and NO conversion in the absence of the major endogenous NO generating system.

It is important to emphasize that the experiments in both **study I & V** were carried out under normoxic conditions and therefore the higher potency of the nitrate-nitrite-NO pathway cannot be explained simply by changes in oxygen tension. It has been suggested that under hypoxic conditions the eNOS mediated NO production which relies on oxygen becomes dysfunctional while the nitrate-nitrite-NO pathway is instead more active [167]. This is undoubtedly true but based on our recent results, we propose the existence of an endogenous 'competition or crosstalk' between these two different NO generating systems irrespective of the oxygen tension. As a general feature, it seems that higher doses of inorganic nitrate inhibit eNOS activity whereas conditions where eNOS is genetically or pharmacologically inactivated are associated with higher potency of the nitrate-nitrite-NO pathway. From a chemical perspective it is interesting to note that nitrite can readily be converted to highly bioactive nitrogen species both through a one-electon reduction (forming NO) and oxidation (forming the NO_2 radical) [168]. Such intricate chemistry may help to explain some of the differential effect of nitrite under normoxic versus hypoxic conditions.

4.2.3 The effects of inorganic nitrate are stronger in aged rats characterized by lower eNOS activity (study IV)

Similarly to eNOS $^{-/-}$ mice, geriatric wt rodents, which constitute a more natural and clinically relevant animal model, are characterized by hypertension, lower eNOS activity and endothelial dysfunction. In particular, the plasma citrulline/arginine ratio, the plasma cGMP levels and the aortic eNOS phosphorylation levels on ser1177 (activation site) were significantly lower in older rats indicating lower eNOS activity and NO-mediated signaling (fig.3E, F, & S3, **study IV**). Moreover, resistance vessels from these aged rats possess attenuated relaxations to Ach and enhanced contractions to AngII (fig.5A & B, **study IV**) which could be indicative of eNOS mediated endothelial dysfunction according to previously published results [169, 170] Administration of a high dose of inorganic nitrate (10 mM) for 14 days significantly reduced blood pressure, improved their metabolic phenotype, increased the plasma cGMP levels and normalized the dilatory and contractile responses in mesenteric arteries in old but not in young animals (fig.1D-F, 2, 3F, 5A & B, **study IV**). Natural aging is a more stochastic model with many other cofounding factors compared to pharmacologic or genetic eNOS inactivation. Therefore, one should be more cautious and not interpret the higher potency of nitrate in the aged animals solely as a result of reversed eNOS dysfunction. Nevertheless, the fact that the nitrate mediated effects were observed only in old

rats which have compromised eNOS function, further supports and confirms the findings from **studies I & V**.

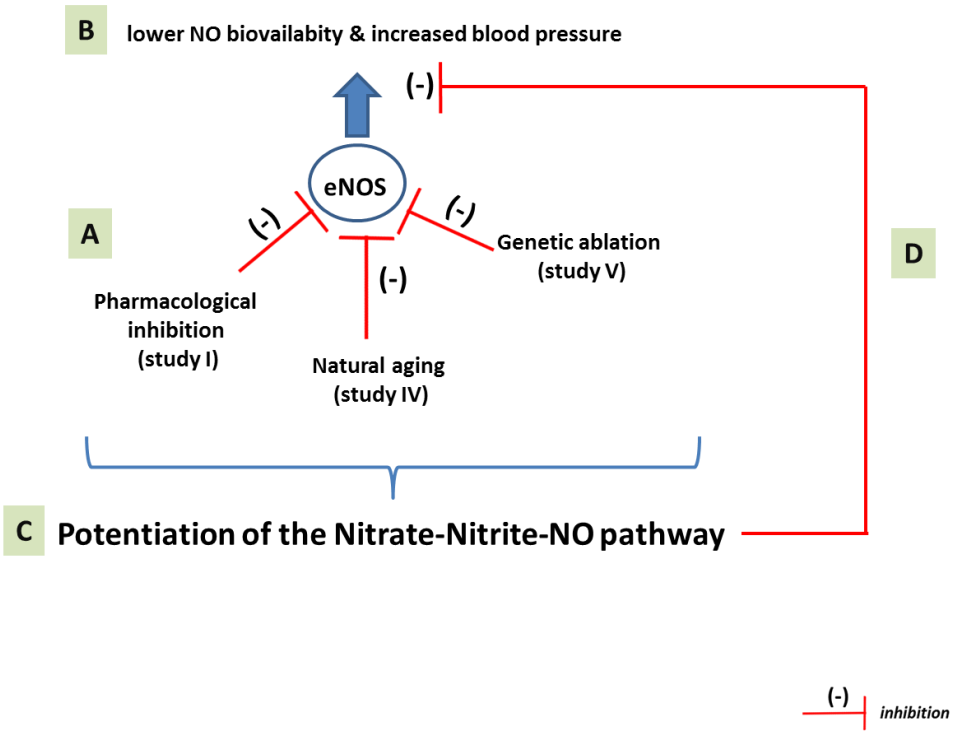

Figure 7: *Summarized overview of studies I, IV& V. A) Phramacological inhibition of eNOS, natural aging or genetic ablation of eNOS lead to reduced eNOS activity. B) The reduction in eNOS activity lowers NO bioavailability and therefore increases blood pressure. C) In parallel, these conditions facilitate the activation of the nitrate-nitrite-NO pathway. D) The nitrate and nitrite derived NO contributes to an improvement of the disrupted NO bioavailability and reduces the abnormally high blood pressure.*

4.2.4 Nitrite reduces macrophage NOX activity in a NOS independent manner with a simultaneous downregulation of iNOS gene expression and peroxynitrite formation (study II)

Although in study **II**, our main focus was not on the association between iNOS and nitrite mediated NO production in macrophages, we again made some interesting observations related to the two different NO generating systems. First, inorganic nitrite attenuates LPS induced NOX activity (~40 %) but when the same experiment is repeated in the presence of L-NAME the attenuating ability of nitrite is not lost but is actually stronger (~90-100% reduction vs the LPS+L-NAME treated macrophages) indicating again a similar mechanism as in **study I,** despite the fact that we used a different experimental approach (fig.1C and 2B, **study II**). Moreover, the LPS+NO_2^- treated macrophages were capable of producing NO (in terms of DAF-FM fluorescence) in the presence of L-NAME confirming NOS synthase independent NO production from inorganic nitrite (fig.3B, **study II**). Macrophages express both eNOS and iNOS but the latter is highly expressed and induced upon LPS activation. Therefore, despite the fact that L-NAME is a general NOS inhibitor, we believe that under these particular experimental conditions most of the L-NAME effects are due to iNOS inhibition [171, 172].
Finally, the presence of nitrite in LPS activated macrophages led to a significant reduction in iNOS gene expression and peroxynitrite production which could potentially imply lower iNOS activation (fig. 5B & C, **study II**). However, since we did not measure iNOS protein

expression and activity or use cells where iNOS was selectively blocked, more experiments are required in order to fully understand the correlation between the iNOS and nitrite-derived NO production in innate immune cells.

4.3 Xanthine Oxidoreductase is a major regulator of the nitrate-nitrite-NO pathway (studies I, II and V)

4.3.1 The vasodilatory effect of nitrite on renal afferent arterioles is lost upon XOR inhibition (study I)

Research over the last year has indicated that the vasodilatory effects of nitrite might be reversed in the presence of XOR inhibitors especially under conditions of acidosis and hypoxia which are known to enhance nitrite reduction to NO [90, 173-176]. However, there are groups suggesting that other nitrite reducing systems such as deoxyhemoglobin or carbonic anhydrase might be of greater importance [177-180]. XOR is an enzyme capable of catalyzing the reduction of both nitrate to nitrite and nitrite to NO. The first findings from pure biochemical assays indicated that these reactions are highly favored under low pH and oxygen levels, but later on it was found that they can also take place under normoxic and physiological conditions [86-88, 181].

In **study I**, we found that XOR mRNA is highly expressed in both the renal cortex and in PG-VSMC (Data Supplement) and that pretreatment of the renal afferent arterioles with the XOR inhibitor oxypurinol abolished the vasodilatory effect of nitrite on the AngII mediated contractions under normoxic conditions (fig 2C & D, **study I**). Since the *ex vivo* experiments on renal afferent arterioles are conducted in the absence of any erythrocytes the importance of deoxyhemoglobin as a nitrite reductase *in vivo* cannot be totally excluded. However, in the afferent arterioles one would expect predominantly the presence of oxygenated hemoglobin which is not an efficient nitrite reductase [179] and therefore it is likely that XOR is a major reducer of nitrite to NO in this type of vessels.

4.3.2 XOR inhibition blocks the nitrite-mediated reduction of NOX activity (study I and II)

In agreement with the findings on renal afferent arterioles, the use of the selective XOR inhibitor febuxostat [182] blocked the effects of nitrite on NADPH oxidase activity in both PG-VSMC (**study I**) and in peritoneal macrophages (**study II**). In particular, although nitrite significantly reduced NOX mediated superoxide production this effect was abolished when the cells were pretreated with febuxostat (fig 4D, **study I** and figure 4B, **study II**). These experiments were again carried out under normoxic conditions which supports the hypothesis that XOR is an important regulator of nitrite's action not only under hypoxia but also at physiological oxygen levels.

4.3.3 Enhanced XOR activity in eNOS$^{-/-}$ mice: effects on blood pressure, ROS and NO homeostasis (study V)

Our overall impression from **studies I, II & IV** was that: a) the nitrate-nitrite-NO pathway is more potent under conditions of reduced eNOS activity and b) that XOR is an important regulator of this pathway even under normoxic conditions. Considering our recent findings indicating a crosstalk between the two major NO generating systems we next wondered if there is actually a crosstalk between eNOS and XOR in regulating nitrate, nitrite, NO and ROS homeostasis [162]. In all the previous studies the function of XOR was studied by using pharmacological inhibitors e.g oxypurinol or febuxostat. XOR is a rather complex enzyme capable of producing simultaneously many different products such as uric acid, hydrogen peroxide, superoxide, nitrite and NO and the evaluation of its activity in relation to all these parameters in tissue extracts or *in vivo* is a complicated issue. However, such an evaluation is a necessity if we want to clarify the physiological role of this enzyme in conditions where eNOS function is compromised and this was our main intention in **study V**.

Our initial motivation for this study was the observation that the eNOS$^{-/-}$ mice, despite their hypertensive phenotype, have completely normal plasma levels of nitrate and nitrite, as shown in fig.1A-B **study V**, indicating that a) plasma nitrate and nitrite do not necessarily reflect eNOS activity and b) a compensatory mechanism for the lost eNOS function exists. These findings are in contrast to the general opinion that the plasma nitrate/nitrite are terminal oxidation products of NO and directly reflect NOS activity *in vivo*. Although, earlier publications supported the opposite notion, later and more carefully conducted work indicated that this is not always true and depends heavily on technical parameters alongside with the content of nitrate and nitrite in the diet [161, 183, 184].

The main hypothesis in study **V** was that XOR would be upregulated in eNOS$^{-/-}$ mice making them more responsive to the nitrate-nitrite-NO pathway. Indeed XOR activity and gene expression was upregulated only in the eNOS$^{-/-}$ mice but not in iNOS$^{-/-}$ or nNOS$^{-/-}$(fig.1C-G and fig.S3 A, B, D, E, G, H, J, K, **study V**). The iNOS$^{-/-}$ or nNOS$^{-/-}$ mice had significantly lower plasma nitrate/nitrite (*data not shown*) indicating that there is probably a more 'selective' inverse correlation between XOR and eNOS but not with the two other NOS isoforms. Interestingly, treatment of wt mice with the general NOS inhibitor L-NAME for 1 week, although capable of increasing blood pressure was not associated with XOR upregulation (fig.S3 C, F, I, L, **study V**) or normal plasma nitrate/nitrite (*data not shown*). In contrast, an earlier study in rats suggested that treatment with L-NAME for 6 weeks was associated with higher XOR protein expression and inhibition of this enzyme attenuated to some extent the blood pressure lowering effect of inorganic nitrite [185]. These discrepancies may depend on the duration and dosing of the L-NAME treatment or even species differences and future studies are warranted in order to fully elucidate the differences between the pharmacologic and genetic eNOS inhibition. After this initial screening among the different NOS$^{-/-}$ mice, we next decided to specifically address the differential XOR-dependent sensitivity to the nitrate-nitrite-NO pathway in the wt and eNOS$^{-/-}$ mice. From a series of different experiments we concluded that eNOS$^{-/-}$ mice to a much higher extent convert inorganic nitrate to nitrite and then NO than wt mice and this bioconversion is attenuated in the presence of febuxostat (fig.2 and 3, **study V**). Moreover, chronic treatment with febuxostat for 3 weeks elevated blood pressure even more in eNOS$^{-/-}$ mice but had no effect on in wt (fig.6A-C, **study V**), indicating that even under baseline conditions without any extra addition of dietary nitrate, XOR is an important blood pressure regulator attempting to compensate for the lost eNOS function. In agreement with that, the high pharmacological dose of nitrate (10 mM in the drinking water) significantly decreased blood pressure in

eNOS$^{-/-}$ mice and this effect was abolished by febuxostat while the opposite trend was observed in the wt (fig. 6D-F, **study V**). Finally, from a mechanistic point of view we suggest that inorganic nitrate and nitrite are able to switch the function of XOR, even under normoxic conditions, (fig.4 C-E and fig.5, **study V**) from an oxygen reductase (superoxide generator) to a nitrate and/or nitrite reductase with lower amounts of ROS and higher levels of NO being generated.

In conclusion, we propose that in the absence of eNOS, XOR is upregulated as a compensatory mechanism for the disrupted NO homeostasis. Possibly, eNOS deficient mice may also use other compensatory mechanisms such as a upregulation of iNOS or nNOS or enahnced renal reabsorption of nitrate and nitrite in an attempt to uphold NO homeostasis. Future studies are required in order to understand more the NO and ROS regulation in this context.

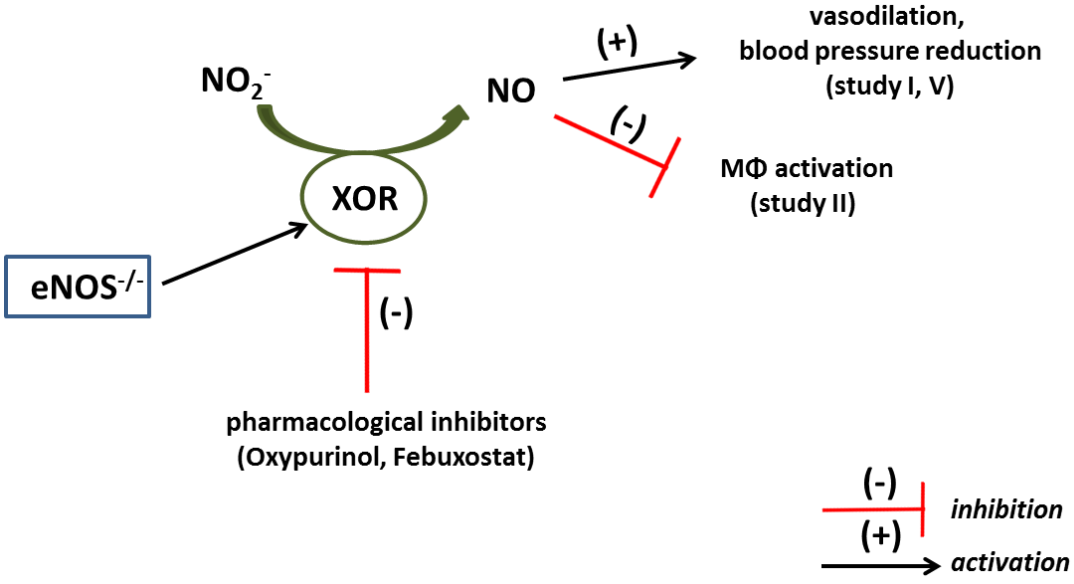

Figure 8: *Summarized overview of the studies I, II & V indicating that XOR is a main regulator and target of the nitrate-nitrite-NO pathway. Moreover, the genetic ablation of eNOS leads to a higher XOR activity which makes the eNOS$^{-/-}$ more sensitive to the nitrate-nitrite-NO pathway. (MΦ: macrophages).*

5 CONCLUSIONS

- In AngII-mediated hypertension dietary nitrate is converted to nitrite by oral commensal bacteria and then to NO by XOR expressed in the renal microvasculature. This nitrite derived NO pathway appears to target NOX in the renal microvasculature. This is associated with lower superoxide production, increased arteriolar dilatation and attenuation of the development of hypertension. (**study I**)

- Inorganic nitrite attenuates NOX activity in activated cells of the innate immune response such as human monocytes and mouse macrophages. The mechanism involves a XOR-mediated reduction to NO, and is associated with a decrease in iNOS gene expression and peroxynitrite production. (**study II**)

- Nitrate mediated reduction of NOX activity might be associated with a simultaneous activation of the AMPK kinase in the liver leading to improved glucose clearance in a mouse model of metabolic syndrome. (**study III**)

- Dietary nitrate improves age-related hypertension and metabolic abnormalities in aged rats. Mechanistically, nitrate downregulates the elevated NOX-mediated superoxide production in the cardiovascular system and the AngII-mediated contractility in resistance vessels. Moreover, it alters the gene expression of the AngII receptors, towards lower AT_1 and higher AT_2 levels. (**study IV**)

- The effects of both nitrate and nitrite are not only NOS independent (**studies I, II & V**), but they seem to be potentiated when eNOS-mediated NO production is pharmacologically (**study I**), genetically (**study V**) or naturally (**study IV**) impaired.

- Genetic ablation of eNOS is associated with a higher XOR activity that partly compensates for the disrupted NO homeostasis and the elevated blood pressure. Moreover, $eNOS^{-/-}$ mice are more responsive to exogenous dietary nitrate whose bioconversion to nitrite and NO is almost abolished upon XOR inhibition. (**study V**)

6 GENERAL DISCUSSION & FUTURE PERSPECTIVES

Although first described back in 1994, many aspects of the nitrate-nitrite-NO pathway, such as its relation to the enzymes producing ROS or the classical NOS-dependent NO production, still remain unclear. Moreover, although the effects of this pathway in the cardiovascular system have been extensively studied its role on the immune cells remains largely unknown.

In the current thesis we tried to uncover the answers to some of these questions by using a broad spectrum of experimental approaches from pure biochemical assays to studies in cells and whole animals. Overall our results indicate that NOX and XOR, which are two major sources of ROS in mammalian cells, are also targets and possible regulators of the nitrate-nitrite-NO pathway in different models of cardiovascular and metabolic disorders and in innate immune cell response.

These findings are in agreement with previous publications indicating that nitrate and nitrite can attenuate oxidative stress and inflammation, but the mechanism or the main targets of this pathway, especially under normoxia, had not been elucidated [132, 186-188]. We believe that the results presented herein give new insights into the nitrate-nitrite-NO pathway and open new scientific questions relevant from basic to clinical research levels. For example, although it becomes evident from **studies I-IV** that NOX activity is strongly downregulated by nitrate and nitrite, via a mechanism that involves NO production, the exact details of the mechanism have not been fully clarified yet. One big obstacle for deep molecular studies is the lack of specific antibodies or pharmacological inhibitors of the different NOX subunits [189]. Another problem in studying NOX is that studies with purified enzyme are difficult since it consists of many cytoplasmic and membrane-associated subunits.

Based on the results of the current thesis we can conclude that the reduction of NOX activity with nitrate (*in vivo*) or nitrite (*in vitro*) cannot be explained by any major changes at the mRNA level (no changes in **studies I** and **II,** lower gene expression only for p22phox and NOX4 in **study IV**) or because of direct scavenging of superoxide by NO since the levels of peroxynitrite in **study II** were actually lower and acute treatment with nitrite had no effect on the LPS activated macrophages (*data not shown*). However, in order to fully exclude the possibility of changes at the mRNA level or any direct scavenging effect detailed time course experiments should be conducted. According to previous literature, NO can attenuate NOX activity via mechanisms that involve post-translational modifications mediated by e.g S-nitrosylation, signaling changes that could reduce the translocation of the cytosolic subunits to the cell membrane or lower transcriptional expression of the NOX subunits [190-192]. Interestingly, on a very recent follow-up study on macrophages we confirmed again the findings from **study II** and we also observed that nitrite reduces the protein expression of the cytosolic subunits p47phox and p67phox via an undefined mechanism [193].

It remains to be elucidated if all actions of nitrate and nitrite on NOX function are mediated via NO *per se* or via other bioactive nitrogen species. The fact that nitrate and nitrite are rather ineffective either on healthy animals, on control untreated cells or in vessels pretreated with a NOX inhibitor might indicate that the degree of activation of this enzyme somehow also regulates the efficiency of the nitrate-nitrite-NO pathway. One fascinating possibility could be that NOX itself is a new unidentified nitrite reductase since this enzyme has a heme moiety in its catalytic center and in theory heme could potentiate the reduction of nitrite to NO or HNO [194]. However, since most of the effects of nitrate and nitrite are blocked by febuxostat which is a selective XOR inhibitor any contribution of NOX as a reductase would be rather low. However, this might be different in disease conditions when NOX activity is

increased. Another possibility is that NOX might be a central regulator of the cellular uptake of nitrite and/or nitrate via a redox sensitive mechanism that triggers the uptake of these anions in conditions of higher superoxide production. So far there is very limited knowledge whether the mammalian cells express nitrate or nitrite transporters or channels and how these could be regulated. An important study showed that at least in the salivary glands and fibroblasts from humans and pigs, Sialin is a protein that can function as a nitrate transporter [195]. Moreover, exogenously administrated inorganic nitrate is highly reabsorbed (~85-97%) in the renal tubules of dogs which could possibly indicate that this anion could be of physiological significance [196]. In addition, inorganic nitrite has also been shown to be reabsorbed in the proximal tubule via a mechanism that involves carbonic anhydrase and AQP1 in humans [197]. Moreover, it was found that AQP6 is a specific nitrate transporter in human kidney embryonic cells [198]. Interestingly, other publications report a correlation between aquaporins and NOXs, which could be of interest considering that the latter are highly inhibited by the nitrate-nitrite-NO pathway. For example, in rat aortic VSMC AQP1 is used as a channel for the intracellular uptake of hydrogen peroxide at pathophysiological levels, which in turn activates NOX-mediated superoxide production [199]. Moreover, neurons from epileptic individuals are characterized by higher NOX2-mediated superoxide production that targets post-translationally AQP4 and alters H_2O and ions balance [200]. Whether different kind of aquaporins are used in different cell types for the intracellular uptake of nitrite and/or nitrate and how exactly this is related to the effects of these anions on NOX function remains to be elucidated. However, we believe that such an investigation could open up possibilities for new therapeutic interventions that lead to higher tissue selectivity and efficiency of the nitrate-nitrite-NO pathway.

Another finding of the present thesis that creates many new questions is the observed XOR upregulation in the $eNOS^{-/-}$, which may have important implications on ROS and NO homeostasis, especially in conditions with compromised eNOS function. Combined with our recent findings suggesting the existence of a crosstalk [162] between the NOS system and the nitrate-nitrite-NO pathway, the results from **study V** reinforce the existence of such a crosstalk between XOR and eNOS for NO production and blood pressure regulation. It remains to be elucidated why only the eNOS deficient mice have an upregulated XOR activity with simultaneous normal plasma nitrate and nitrite levels. Moreover, the exact nature of a proposed XOR-eNOS crosstalk is not known, and warrants future investigations. One possibility is that eNOS-mediated signaling could downregulate XOR expression and activity and vice versa. In addition, it would be interesting to clarify if there is any physical interaction between the two enzymes at the cellular level and how this interaction could modify their activity during low and high nitrate or nitrite concentrations. Two older publications suggested a physical interaction and crosstalk between nNOS and XOR in the cardiomyocytes of the failing heart [201, 202]. These authors considered XOR as a pure source of superoxide and did not make any associations with its nitrate and nitrite reductase activity. Whether something similar happens in eNOS expressing cells and how this could be associated to the NO and ROS balance should be clarified in the future. In addition, it would be of interest to see if chronic use of XOR inhibitors could actually be associated with detrimental cardiovascular effects in patients with endothelial dysfunction, due to abrogation of the nitrate-nitrite-NO pathway. Actually, febuxostat has been used in two recent phase II-IV clinical trials on patients with mild to moderate hypertension and hyperuricemia (identifier number: NCT01496469, NCT01328769) where the primary outcome was the measurement of blood pressure and renal blood flow respectively. Interestingly, in the first study there were no significant differences with the placebo group while in the second study the febuxostat treated patients actually had almost four times lower renal blood flow at the end of the study. Hyperuricemia has been linked with the development of hypertension [203]. However, two recent clinical trials in hyperuremic patients reported no significant reduction

in blood pressure after treatment with febuxostat [204, 205]. Overall, the results from these studies challenge the wide spread hypothesis that inhibition of XOR would lead to a blood pressure reduction because of lower ROS and uric acid levels. Importantly, all these studies were performed in patients with already pathological levels of uric acid which could possibly contribute to their cardiovascular complications [206]. It would be interesting to test the effects of febuxostat in patients with endothelial dysfunction, hypertension and relatively normal plasma uric acid levels and see if there are similarities with our findings.

Finally, to the best of our knowledge the results in **study II** were the first ones showing a direct effect of nitrite on macrophages, which are crucial for the innate immune response. Previous work conducted in our group and others has proven that the nitrate-nitrite-NO pathway can reduce renal inflammation, leukocyte recruitment in microvascular inflammation and NSAID-induced intestinal injury and LPS-mediated morbidity and mortality in mice, but none of these earlier studies investigated the exact mechanism in specific immune cells [141, 187, 188] We believe that further investigations regarding the effects of the nitrate-nitrite-NO pathway on the immune system as a whole *in vivo* and on different immune cell types *in vitro* is of great importance since there is limited knowledge in this area, and this could have many therapeutic implications. For example, it would be exciting to investigate if nitrate and nitrite are able to interfere with the iNOS-mediated NO production since we have evidence from the current thesis and from a recent publication in our group that nitrite can reduce iNOS expression at both the mRNA (**study II**) and protein level [142]. These effects of inorganic nitrite on iNOS could be of particular interest in sepsis where sustained (over 10 hours) activation of iNOS leads to prolonged vasodilation, hypotension and finally multiple organ damage and death. Unfortunately, a large phase III clinical trial where sepsis patients were treated with a general NOS inhibitor, failed and led to higher mortality rates possibly because of adverse cardiovascular outcomes [207]. After these disappointing outcomes, a new trend has started emerging suggesting that a moderate boost of NO signaling in sepsis could be actually beneficial if parameters such as the dosing and timing of the NO-donating molecules are carefully taken into consideration [208]. In this concept, inorganic nitrite in a dose that produces moderate levels of NO and simultaneously downregulates iNOS can be a promising candidate for treating sepsis. Our results on the macrophages and NOX activity in combination with older findings from our group suggesting that dietary nitrate can downregulate myeloperoxidase (MPO-another important source of ROS in the immune cells) [142] are possibly indicative of a switched immune cell function. Therefore, future findings could have therapeutic value not only in cardiovascular disease, but actually in several disorders characterized by oxidative stress and progressive inflammation.

A summarized overview of all the proposed mechanisms is given on the following fig.9:

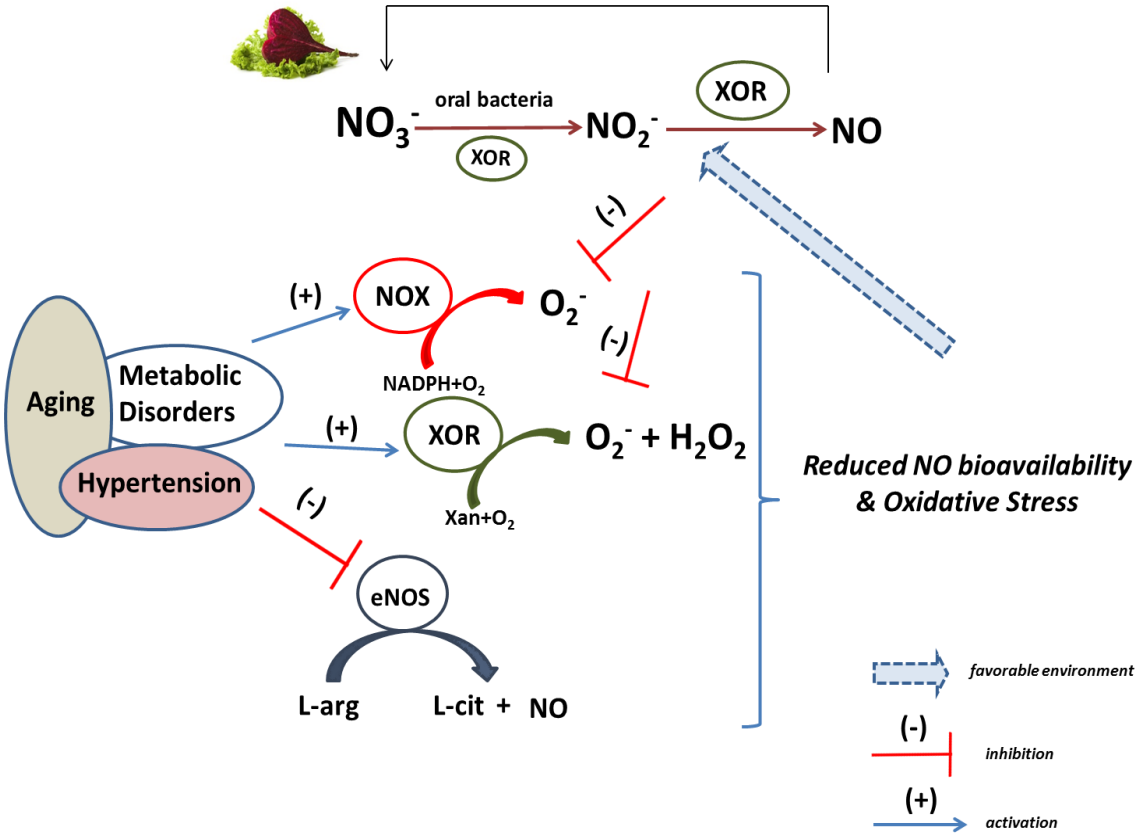

Figure 9: Proposed mechanisms

Aging, hypertension and metabolic disorders are related to each other and characterized by reduced NO bioavailability and oxidative stress. Lower eNOS-derived NO production with a simultaneous elevation of XOR and NOX-mediated ROS production can contribute to the progression of these pathologies. Under such conditions, the production of NO from inorganic nitrate and nitrite is potentiated. Inorganic nitrate received either from the diet (e.g beetroots or green leafy vegetables) or from the oxidation of NO in the circulation is serially reduced to nitrite by oral commensal bacteria and to some extent by XOR. The subsequent reduction of nitrite to NO relies mostly on XOR. NOXs constitute a major target of the nitrate-nitrite-NO pathway that strongly inhibits superoxide production in various cells and tissue types. Genetic ablation of eNOS is associated with higher endogenous XOR activity that potentiates the conversion of nitrate to nitrite and finally to NO and upholds to some extent the dysregulated blood pressure, NO and ROS homeostasis.

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