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EUKARYOTIC AND PROKARYOTIC NITRIC OXIDE SYNTHASES – STRUCTURE-FUNCTION STUDIES

PhD Thesis

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Abstrakt:

Oxid dusnatý (NO) patří mezi nejdůležitější signální molekuly organismů, kde hraje zásadní roli v širokém spektru fyziologických a patologických procesů, včetně vazodilatace, přenosu nervového signálu a imunitní odpovědi organismu. Tato plynná molekula je produktem oxidační reakce katalyzované rodinou syntáz oxidu dusnatého (NOS).U savců byly identifikovány tři izoformy NOS, endoteliální (eNOS), neuronální (nNOS) a inducibilní, nebo také imunologická (iNOS).

Některé bakterie nesou geny kódující proteiny homologní s oxygenázovou doménou savčích NOS a vykazující NO-syntázovou aktivitu *in vitro*. Předpokládá se, že NO tvořený patogenními organismy jako je *B. anthracis* nebo *S. aureus* by mohl hrát kritickou roli v patofyziologickém průběhu infekce. Komparativní studie bakteriálních "NOS-like" enzymů a savčích NOS prokázala jejich základní podobnost a zároveň rozdíly mezi jednotlivými bakteriálními proteiny i savčími izoformami při interakcích s různými analogy substrátu L-argininu a dalšími ligandy. Na základě výsledků měření kinetiky zpětné vazby NO byla predikována přítomnost druhého NO-vazebného místa v aktivním centru některých NOS. Dále byla popsána regulace dynamiky a uvolňování NO z proteinu NOS pomocí sítě vodíkových vazeb mezi hemovou prostetickou skupinou, substrátem a kofaktorem BH_4.

Nitrity (NO_2^-) coby metabolity NO mohou být zároveň jeho zdrojem při nedostatku kyslíku, kdy neprobíhá syntéza NO z argininu. V této práci bylo prokázáno, *in vitro* a *in vivo*, že eNOS, jako jediná ze tří izoforem NOS, vykazuje nitritreduktázovou aktivitu při snížené hladině i kompletní absenci kyslíku, při fyziologickém pH a koncentraci nitritů, za využití alternativní, na argininu a kyslíku nezávislé biosyntetické dráhy.

NO má v mitochondriích důležitou roli při vzniku volných kyslíkových radikálů a v regulaci energetického metabolismu. Existence mitochondriální izoformy NOS je předmětem intenzivního výzkumu a dosud nebyla plnohodnotně prokázána. Nově popsaný rostlinný protein AtNOS1, jehož analogy byly nalezeny v řadě bakterií i eukaryot, byl identifikován jako potenciální NOS, bez jakékoliv podobnosti s dosud popsanými NOS.

Výsledky této práce popřely přímou účast savčích ortologů AtNOS1 na produkci NO. Protein byl lokalizován v mitochondriích a byla prokázána jeho funkce v asemblaci mitochondriálního ribozomu a tím i syntéze mitochondriálních proteinů a základních mitochondriálních funkcích včetně syntézy ATP a apoptózy.

Klíčová slova: Oxid dusnatý, NO, syntáza oxidu dusnatého, nitrit, redukce nitritů, bakteriální NOS-like protein, AtNOS1, AtNOA1, mitochondriální NOS

Abstract*:*

Nitric oxide (NO) is an important signaling molecule in organisms. It plays a role in wide spectrum of physiological and pathophysiological processes, including vasodilatation, neurotransmission and host defense. The gaseous molecule of NO is produced by oxidative reaction catalyzed by proteins from the family of nitric oxide synthases (NOSs). Three NOS isoforms were identified in mammals, endothelial (eNOS), neuronal (nNOS) and inducible or immunologic (iNOS).

Some bacteria harbor genes coding for proteins homologous to the mammalian NOS oxygenase domain and showing NO-producing activity *in vitro.* NO generated by pathologic organisms such as *B. anthracis* and *S. aureus* is supposed to play a critical role in the pathophysiological processes during the infection. Comparative study of bacterial NOS-like proteins and mammalian NOSs confirmed their principal similarity, but also revealed differences in the interactions of distinct bacterial proteins and mammalian NOS isoforms with different analogs of substrate L-arginine and various ligands. On the basis of the kinetics measurement of NO-rebinding a second NO-binding site in the active center of NOS was predicted. Further, the regulation of NO dynamic and release from the protein by the active site H-bonding network connecting the heme, the substrate and BH_4 cofactor was described.

Nitrite (NO_2^-) as a NO metabolite can also be its source by the absence of oxygen, when the arginine-dependent NO production is stopped. This work proved both *in vitro* and *in vivo* that eNOS is the only NOS isoform capable of nitrite reduction activity at physiological pH and nitrite levels, under oxygen levels from normoxia to complete anoxic conditions, by using a new, arginine- and oxygen-independent NO-producing pathway.

NO in mitochondria plays an important role in reactive oxygen species (ROS) formation and in the regulation of energetic metabolism. The existence of mitochondrial NOS isoform is subject to an extensive research, but the full proof is still missing. Newly described plant protein AtNOS1, with orthologs in bacteria and eukaryotic organisms, was identified as a putative novel NOS. The results of this work denied direct involvement of mammalian AtNOS1 orthologs in the NO production. The protein was localized in mitochondria and its function was shown to take place in the mitochondrial ribosome assembly, mitochondrial protein synthesis, and such basal mitochondrial functions as ATP synthesis and apoptosis.

Keywords: Nitric oxide, NO, nitric oxide synthase, NOS, nitrite, nitrite reduction, bacterial NOS-like proteins, AtNOS1, AtNOA1, mitochondrial NOS

„Success is the ability to go from failure to failure without losing your enthusiasm.“

Sir Winston Churchill

„Když nemůžeš, přidej!“

Emil Zátopek

Contents

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List of original communications

This thesis is based on the following publications. Impact factors (IF) and citations frequency as of June 2010 is shown

Mikula I, Durocher S, Martasek P, Mutus B, Slama-Schwok A. Isoform-specific differences in the nitrite reductase activity of nitric oxide synthases under hypoxia. Biochem J. 2009 Mar 15;418(3):673-82.
IF = 5.155, 4 citations

Salard I, Mercey E, Rekka E, Boucher JL, Nioche P, **Mikula I**, Martasek P, Raman CS, Mansuy D. Analogies and surprising differences between recombinant nitric oxide synthase-like proteins from Staphylococcus aureus and Bacillus anthracis in their interactions with l-arginine analogs and iron ligands. J Inorg Biochem. 2006 Dec;100(12):2024-33. Epub 2006 Sep 28.
IF = 3.252, 6 citations

Zemojtel T, Fröhlich A, Palmieri MC, Kolanczyk M, **Mikula I**, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, Durner J. Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. 2006 Nov;11(11):524-5; author reply 526-8. Epub 2006 Oct 9.
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List of Abbreviations

ACE I	autoinhibitory control element I
ACE II	autoinhibitory control element II
AtNOS1	nitric oxide synthase from A. thaliana
BA-NOS	NOS-like from B. anthracis
BH_4	tetrahydrobiopterin
bsNOS	NOS-like from B. subtilis
Ca	calcium
Ca^{2+}/CaM	calcium-activated calmodulin
CaM	calmodulin
cGMP	cyclic guanosine monophosphate
cGTP	cyclic guanosine triphosphate
CYPOR	NADPH-dependent cytochrome P450 oxidoreductase
deiNOS	NOS-like protein from D. radiodurans
EDRF	endothelium-derived relaxating factor
EGFP	enhanced green fluorescent protein
eNOS	endothelial NOS
EPR	electron paramagnetic resonance
FAD	*flavin adenine dinucleotide*
FMN	flavin mononucleotide
FNR	ferredoxin NADP+-reductase
GDP	*guanosine* diphosphate
G-proteins	guanine nucleotide-binding proteins
gsNOS	NOS-like protein from G. stearothermophilus
GTP	guanosine triphosphate
GTPase	GTP hydrolase
HDMECs	human dermal microvascular endothelial cells
HNS	heme domain of NO Synthase-like proteins
Homo-L-arg	homo-L-arginine
Hsp90	heat shock protein 90
Im	imidazole
iNOS	immunologic (or inducible) NOS
iNOSoxy	oxygenase domain of iNOS
L-Arg	L-arginine
L-NMA	L-*N*-methylarginine
mAtNOS1	murine AtNOS1
mtNOS	mitochondrial nitric oxide synthase
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of NADP
NIO	N-iminoethyl-L-ornithine
NMDA	N-methyl-D-aspartate
NMMA	N-methyl-L-arginine
nNOS	neuronal NOS
NO	nitric oxide
NO_2^-	nitrite

NO_2-L-Arg	NO2-L-arginine
NOA1	NO-associated protein 1
NOCNOS	nitric oxide synthase from Nocardia sp.
L-NOHA	Nω-hydroxy-L-arginine
Nor-L-arg	Nor-L-arginine
NOS	nitric oxide synthase
NOSIP	eNOS interacting protein
NOSTRIN	eNOS traffic inducer
O_2	oxygen
$O_2^{-\cdot}$	superoxide
$ONOO^-$	peroxonitrite
OXPHOS	Oxidative phosphorylation
PDZ	PSD/Disc-large/ZO-1 homologous
ROS	reactive oxygen species
RSNOs	S-nitrosothiols
SA-NOS	NOS-like protein from S. aureus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEITU	S-ethylisothiourea
sGC	soluble guanylate cyclase
TEM	transmission electron microscopy
TGC	trophoblast giant cell
THF	tetrahydrofolate
tRNA	transfer ribonucleic acid
XO	xanthine oxidase
XOR	xanthine oxidoreductase

Review of the Literature

History

The first known report showing the beneficial properties of nitric oxide (NO), even though he did not know that the active compound was NO, was in 1867, when Brunton described the use of nitrogen compound amyl nitrite and later nitroglycerin as a treatment for the patients with angina pectoris (*Brunton (1867)*). In 1916 it was discovered that the nitrite uptake in the mammals exceeds its secretion (*Mitchell et al. (1916)*). In 1977 Murad et al. described the vasodilatory effect of nitric oxide on the activation of soluble guanylate cyclase cascade (*Arnold et al. (1977)*). Later, in 1980, Furchgott and Zawadski found out the involvement of endothelial cells in the smooth muscle blood-vessel cells relaxation (*Furchgott et al. (1982)*), and named the factor responsible for this effect an endothelium-derived relaxing factor (EDRF) without knowing its identity. Finally, in 1987 the EDRF was identified as NO by Ignarro and coworkers (*Ignarro et al. (1987); Palmer et al. (1987*)) which started a great scientific interest in this molecule.

After the identification of NO, the biochemistry of NO formation from the amino acid L-arginine and oxygen to L-citrulline catalyzed in the organisms by the enzymes family called nitric oxide synthases was reported. Three scientists – Furchgott, Ignarro and Murad - that stood at the beginning of this incredibly fast-growing scientific field (over 100.000 entries regarding nitric oxide and over 50.000 regarding nitric oxide synthase in PubMed) were in 1998 awarded Nobel Prize for medicine for their contribution to discovering the role of nitric oxide as a signaling molecule in cardiovascular system.

An irony of history is a fact that the founder of Nobel Prize Alfred Nobel was offered a nitroglycerin (a compound that he used for his invention of dynamite) by his physician for his chest pain. Nobel was too afraid of using it, so he refused and later died of a heart attack.

Nitric oxide

Nitric oxide is a lipophilic, diatomic gaseous molecule with one unpaired valence electron and very short biological half-life (3-5 seconds). The complexity of its biological activity in the organisms results in large amount of potential interactions with various compounds such as reactive oxygen compounds, metal ions and proteins. NO reacts with molecular oxygen forming nitrogen dioxide (NO_2), a radical with higher reactivity and toxicity compared to NO (*Ford et al. (1993); Fukuto et al. (2000)).* A product of the reaction of NO with superoxide ($O_2^{-\cdot}$) is peroxynitrite ($ONOO^{-\cdot}$), a potent oxidative agent. These substances belong to the family of oxygen derivatives and are very important in vascular biology because of their redox potential. The disturbance of the oxidative-reductive balance results in oxidative stress, which is associated with oxidative damage and inflammation in cardiovascular diseases, including diabetes and hypertension (*Touyz et al. (2004*)).

NO is an unstable free radical, a signaling molecule in the blood-vessels, immunological and central nervous systems. It's freely diffusible, soluble, with higher solubility in hydrophobic media then in aqueous solutions. High solubility in lipids leads to increased NO concentration levels in the lipid-rich locations such as cell membranes and lipoproteins (*Lancaster, J.R. (2000)*).

The reactivity of NO under physiological and pathological conditions fully depends on the concentration and location of NO production (Fig. 1). NO is very susceptible to reaction with iron, thus forming a complex in the heme group. Heme is a component of many metalloproteinase, called hemoproteins, e.g. cytochrome P450, catalases, nitric oxide synthases (NOS) and soluble guanylate cyclase (sGC) (*Wink et al. (1993); Cooper (1999)*). The activation of sGC by interaction with NO and consecutive cGMP production is considered to be the main NO-signaling pathway and is well characterized *(Koesling et al. (2000); Reynolds et al. (2000); Stone et al. (1994); Yu et al (1994)).* It leads to the activation of proteinkinases, phosphodiesterases and ion channels (*Chun et al. (2001)*), resulting in different modulation of cell functions such as muscle relaxation, inhibition of platelets aggregation or synaptic plasticity (*Arnold et al. (1977); Furchgott et al. (1980); Fukuto et al. (2000)).*

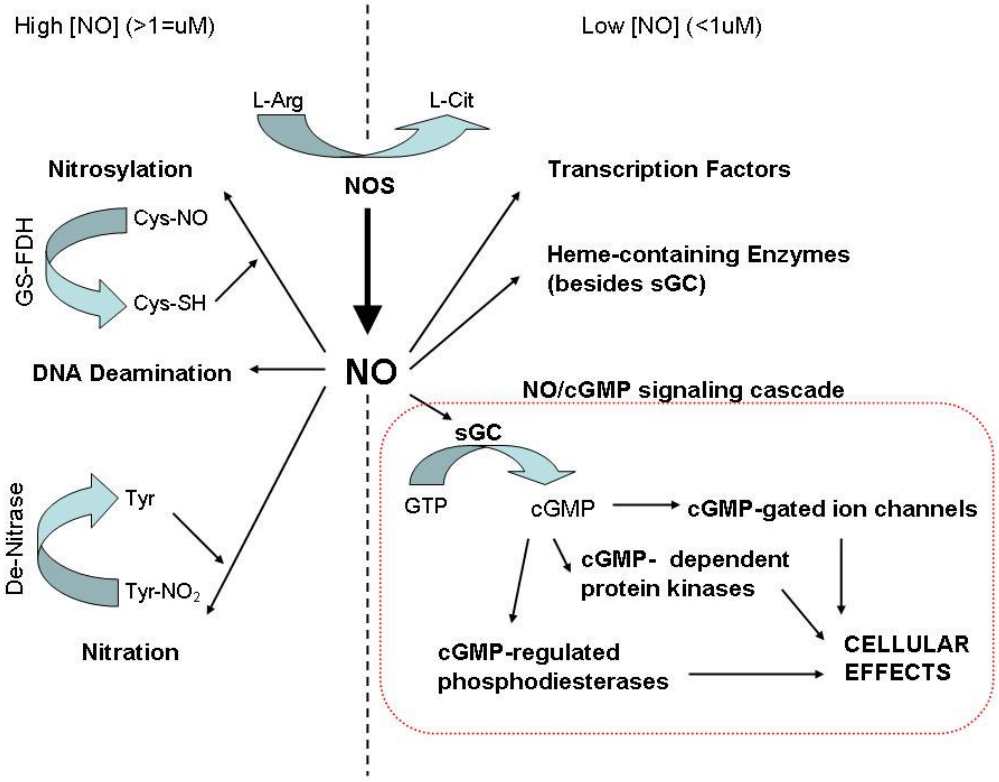

Figure 1. Summary of NO transduction and its effect depending on concentration. The main NO-signaling pathway is considered to be the NO/cGMP signaling cascade (highlighted). (Adapted from *Hanafy et al. (2001) & Friebe and Koesling (2003)*)

The Structure of Nitric Oxide Synthases

Mammalian nitric oxide synthases (NOSs) are large proteins ranging in size from 135 to 160 kDa. The enzyme is only active in the homodimeric form, the monomeric form is inactive. The molecule (subunit) of NOS consists of an N-terminal oxygenase domain, C-terminal reductase domain and calmodulin-binding site in-between. In the active enzyme there is heme as a prosthetic group and several cofactors - tetrahydrobiopterin (BH_4), calmodulin, FMN, FAD and NADPH (Fig. 2). The reductase domain architecture is very similar to that of cytochromes P450 (*Narhi et al. (1987)*).

Nitric oxide synthases utilize molecular oxygen and electrons from NADPH to oxidize the substrate L-arginine into the intermediate OH-L-arginine, which is then oxidized into NO and L-citrulline (*Griffith et al. (1995)*).

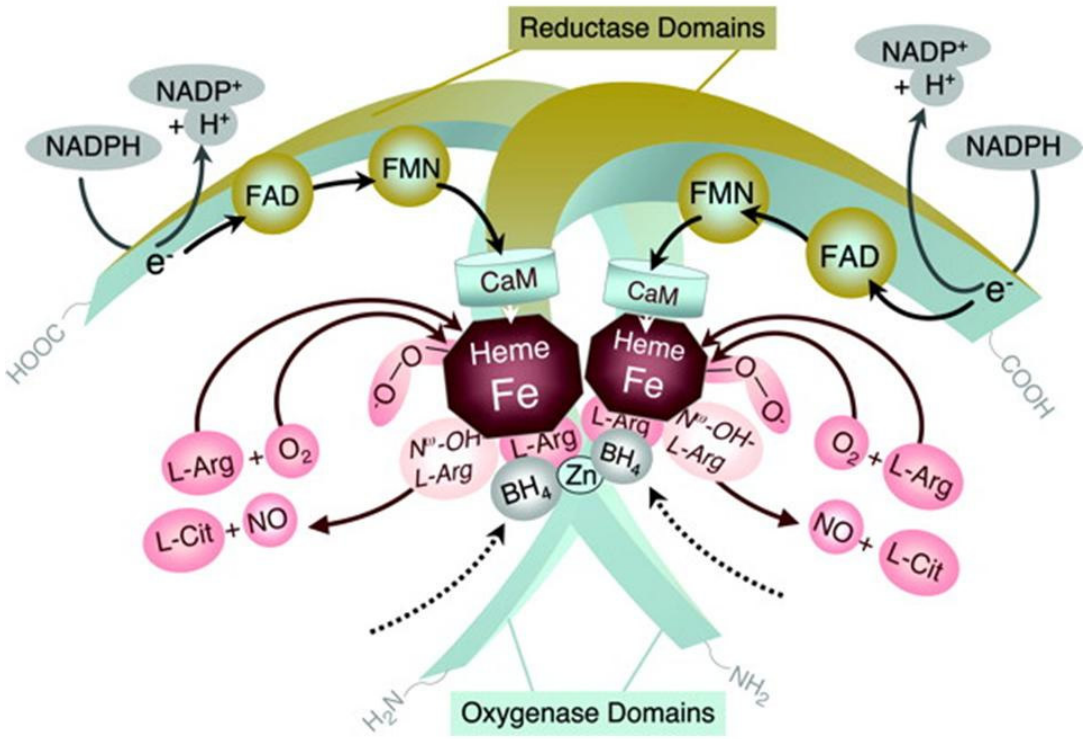

Figure 2. The structure of active NOS homodimer and scheme of NOS catalysis. The binding of Ca^{2+} activated calmodulin stimulates the transfer of electron from NADPH via flavins FAD and FMN within the reductase domain to heme in the active center of the opposite monomer. The cofactor BH_4 and the substrate L-Arg bind only to the protein in the dimeric form. When sufficient substrate L-arginine and cofactor BH_4 are present, NOS couple the heme and O_2 reduction to the synthesis of NO·and L-citrulline via L-NOHA is an intermediate in the reaction. (Adapted from Förstermann and Münzel (2006))

Reductase Domain

The C-terminal part of the protein is the reductase domain. It contains a binding site for the electron donor NADPH as well as the biding sites for two flavin cofactors – FAD and FMN – therefore it is also called flavoprotein domain. The domain structure is very similar to NADPH-dependent cytochrome P450 oxidoreductase (CYPOR); the sequence homology is cca 58% *(Bredt et al. (1991))*.

The reductase domain is divided into two subdomains, each containing one of the flavin cofactors. At the N-terminus there is the FMN-binding subdomain, which is similar to flavodoxin. In the middle of this subdomain of the constitutive NOS isoforms there was found a cca 40 aminoacid-long additional sequence that is not present in iNOS, CYPOR or flavodoxin *(Salerno et al. (1997)*). This insert (called an autoinhibitory

control element I (ACE I)) is though to be an autoinhibitory element. The deletion of ACE I lowered the sensitivity of constitutive NOSs (eNOS and nNOS) to calcium concentration and significantly improved the maximal activity, especially that of eNOS *(Daff et al. (1999)*; *Nishida et al. (1999); Nishida et al. (2001))*.

The C-terminus subunit of the reductase domain contains FAD- and NADPH-binding site as well as autoinhibitory control element II (ACEII). The two binding sites resemble the ferredoxin $NADP^+$-reductase (FNR). Autoinhibitory control element II, consisting of 21-42 aminoacid residues, depending on isoform, is present in all three mammalian NOSs. The ACE II controls electron flow and modulates the Ca^{2+}/calmodulin effect *(Roman, Miller et al. (2000); Roman, Martasek et al. (2000); Lane et al. (2002))*. Both ACE-I and ACE-II are controlled by the phosphorylation, that apparently removes the steric hindrance imparted by these inserts and permits better fidelity of electron flux from the reductase domain to the oxygenase domain *(McCabe et al. (2000))*.

Oxygenase domain

The oxygenase domain, also called heme domain, is located the N-terminal half of the NOS subunit. It contains a heme molecule (iron protoporphyrin IX) as a prosthetic group in the active site, and binding sites for pterin and for the substrate L-Arg *(McMillan et al. (1993))*. Pterin cofactor (BH_4) binding is stabilizing the binding of substrate in the active center and it is present in NOSs, not in cytochromes P450. Despite the similarity between NOS oxygenase domains and P450 in having heme in the catalytic center, the structures show no similarity *(Crane et al. (1998); Fischmann et al. (1999); Raman et al. (1998))*. In contrast to the hydrophobic properties of the channel leading to the active center of P450, the NOS active site shows strong polarity in the substrate pocket *(Poulos et al. (1998))*.

Calmodulin-binding side

The two NOS domains, reductase and oxygenase, are connected by the calmodulin (CaM)-binding region. The binding of calcium-activated calmodulin (Ca^{2+}/CaM) is essential for the activity of the enzyme. It stabilizes the dimer and allows the electron flow from the reductase domain to the oxygenase domain (*Abu-Soud et al. (1994)*. The electrons are transported from NADPH through flavin cofactors FAD and FMN to the heme active center, reducing the heme and thus enabling the NO-producing reaction *(Griffith et al. (1995))*. Importantly, the electron from the reductase domain passes to the oxygenase domain of the opposite dimer subunit, not to that of the subunit to which the NADPH binds.

Another important structural element present in NOS is the zinc tetrathiolate cluster, which contributes to the stabilization of the homodimeric NOS *(Hemmens et al. (2000); Li et al. (1999); Ludwig and Marletta (1999))*. Two cysteines from each heme domain contribute thiol moieties to this cluster. Aside from its structural role, the possibility that S-nitrosation of the zinc tetrathiolate cysteines of iNOS plays a regulatory role by virtue of their importance in homodimer stability and increased nucleophilicity of the catalytic center was proposed *(Mitchell et al. (2005))*.

Mechanism of NO Biosynthesis

NOS catalyzes the transformation of L-Arg to NO and L-citrulline via two step reaction with the N^{ω}-hydroxy-L-arginine (NOHA) as the intermediate (Fig.3).

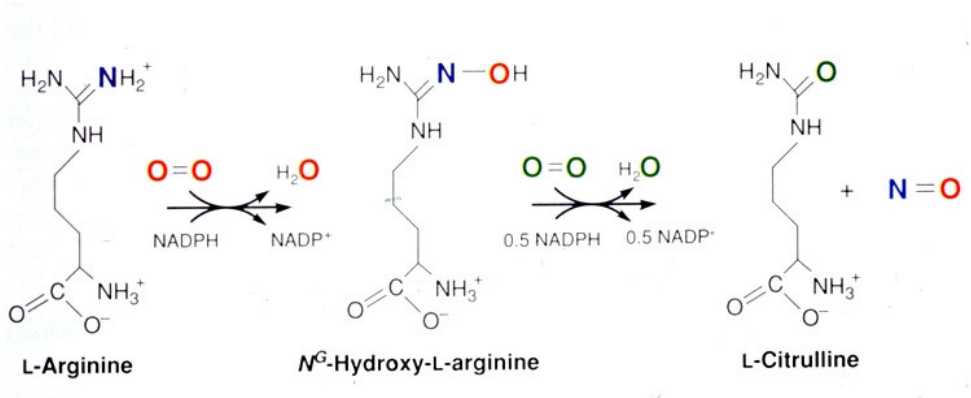

Figure. 3. Production of NO catalyzed by NOS. NO is formed through two sequential monooxygenation reaction. The reaction mechanism is similar to the reaction catalyzed by cytochrome P450 oxidoreductase system.

The first, and the slowest step of the whole reaction, is the electron transfer from NADPH to the ferric heme, provided by flavoprotein domain, thus enabling the formation of ferric hemesuperoxy species (Species I) in the presence of O_2 (*Wei et al. (2003); Ledbetter et al. (1999)*). Species I is not reactive towards Arg but could react with NOHA *(Huang et al. (2001))*. Instead of releasing superoxide, species I receive an electron from pterin cofactor – tetrahydrobiopterin (BH_4) *(Wei et al. (2003)*), forming a heme-peroxo species (species II), which has only been observed in NOS at cryogenic temperature *(Davydov et al. (2002))*. When BH_4 is absent, the electron can also be provided by flavoprotein domain *(Rusche et al. (1998))*. If the substrate is NOHA, then the previously formed BH_4^+ radical is reduced back to BH_4 by the reaction intermediate *(Hurshman et al. (2003))*. The last step is the protonation of species II, linked with water removal, forming the heme iron-oxo species (species III), which is the one that enters the reaction with either L-Arg or NOHA (Fig. 4).

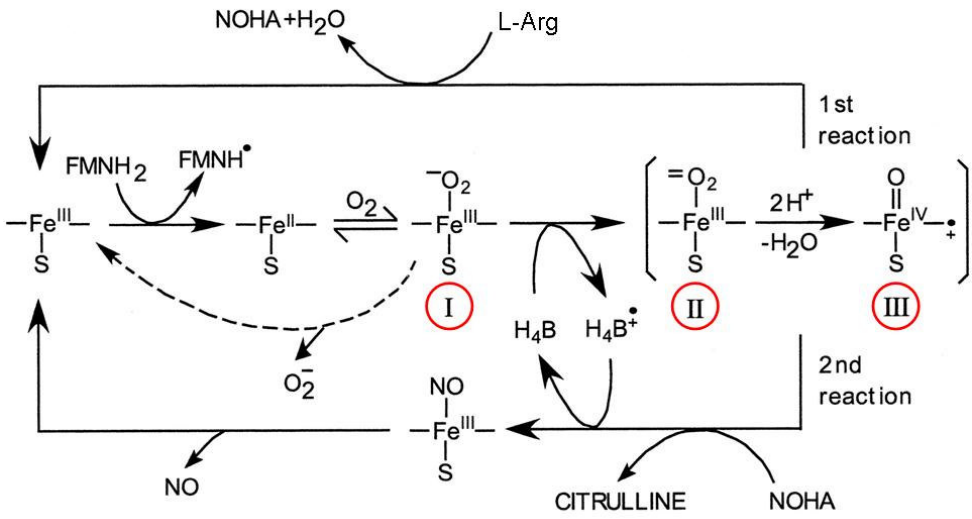

Figure 4. Scheme of NO biosynthesis. The distinct species (I, II and III) are highlighted by circles. (Adapted from Stuehr et al., 2004)

The direct product of the catalysis is not a free NO, but a ferric heme-NO complex *(Negrerie et al. (1999); Scheele et al. (1999))*. The mechanism and kinetics of the NO release from this complex is indeed a very important factor in regulation of NO

production by different NOSs and could be affected by different compounds providing a way to control NO production.

Generally, the ferric heme-NO complex can release the NO directly, but it can also be reduced to the ferrous heme-NO complex by flavoprotein domain. The release of NO from the second is very slow *(Santolini et al. (2001))*. In the presence of O_2 the ferrous heme-NO complex undergoes an oxidative reaction, forming a nitrite molecule instead of NO and regenerating the ferric enzyme. These two mechanisms compete and only the dispensing among them creates the overall kinetics of the NO production by NOS *(Stuehr et al. (2004))*.

The apparent difference of the distinct NOS isoforms activity is in fact a result of the balancing of those mechanisms by each isoform. This balancing is thoroughly dependent on the particular structure of the active side and also on the composition of the active side environment. The specific activities of three mammalian isoforms are well described in order iNOS > nNOS > eNOS. The distribution pattern of the enzyme into five main forms – ferric, ferrous, ferrous-O_2, ferric-NO and ferrous-NO – is the key for the global kinetic mechanism. It also helps us explain why the specific activity of nNOS is lower then iNOS, although the rate of NO biosynthesis for nNOS is twice faster than for iNOS. Despite the very fast conversion of the ferric form to the ferrous, the rate of conversion of ferric-NO to ferrous-NO complex is also very fast, so nNOS occurs mainly in the ferrous-NO form with extremely slow rate of NO release competing with nitrite formation. By contrast, the iNOS kinetic parameters are moderate, resulting in high ferric form distribution, and importantly, higher ferric-NO form distribution than nNOS, which explains the divergence of specific activity values *(Santolini et al. (2001)).*

Another important factor affecting fundamentally the NO production is the external NO, which is already present in solution. This NO can also bind to the heme, shifting the distribution pattern of individual forms. This affects the balance between two possible mechanisms, thus lowering the NO release rate. The effect of NO solution concentration differs significantly among NOS isoforms *(Abu-Soud et al. (2000); Abu-Soud et al. (2001); Abu-Soud et al. (1995))*.

Mammalian NOS Isoforms

In mammals, there are at least three different isoforms of NOS described. They were originally identified and named according to the tissue where they were first isolated - neuronal NOS (nNOS), endothelial NOS (eNOS) and immunologic NOS (iNOS), which was first found in macrophages (Fig. 5). Although isoforms share 50-60 % homology and have common features of bound cofactors, substrate and cosubstrate, each of them is encoded by a different gene. But according to the enzyme commission nomenclature, the proteins share the same EC number 1.14.13.39.

Based on the main mode of function and regulation, we distinguish two types of NOS - constitutive and inducible *(Nathan et al. (1994))*. The activity of constitutive isoforms - nNOS and eNOS - under physiological conditions displays dependence on calcium concentrations that enables reversible binding of calmodulin *(Bredt et al. (1990); Forstermann et al. (1991))*. The inducible isoform iNOS has calmodulin (CaM) bound permanently as an additional subunit and hence iNOS is not regulated by CaM binding, but is under transcriptional control instead *(Cho et al. (1992))*. This partitioning is simplified in the meaning that eNOS and nNOS are constitutive because their activation does not require new enzyme synthesis, but both of them are also inducible primarily under conditions of traumatic or pathological insult when the synthesis of new enzyme occurs.

The intracellular localization of distinct isoforms determines the accessibility to the extracellular stimuli and to the efficiency of NO production *(Nedvetsky et al. (2002); Bredt et al. (2003))*. The mechanisms of tight control of the enzymatic reaction involve multiplex protein-protein interactions, local availability of cofactors and substrate(s) and posttranslational modifications. The particular isoforms are principally tissue specific *(Roman et al. (2002); Bredt et al. (2003)).*

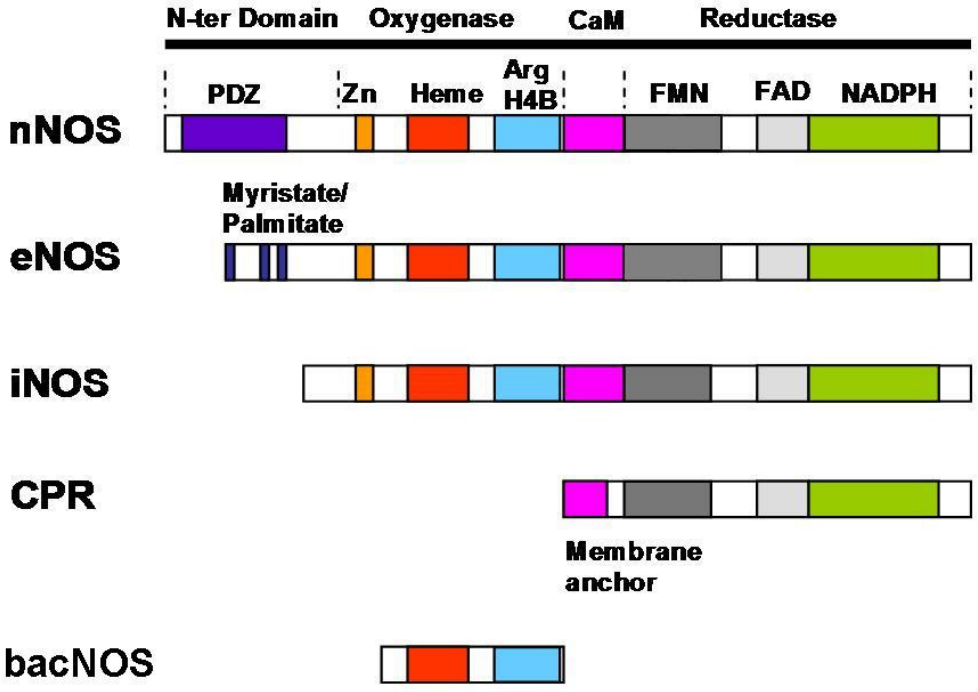

Figure 5. The comparison of eukaryotic NOSs with cytochrome P450 reductase and bacterial NOS-like protein. The individual domains, clusters and binding sites for the heme prosthetic group, cofactors and cosubstrates are shown.

Endothelial NOS

The endothelial isoform - eNOS (or NOS3) - could be considered as the best characterized isoform because of its role in the vascular system. The human *eNOS* gene is located on the chromosome 7 *(Arngrímsson et al. (1997))* and the protein has a molecular mass of 133 kDa**.** It is a constitutive, Ca^{2+}/calmodulin dependent enzyme and takes a role in the vasodilatation and platelets adhesion and aggregation. eNOS is mainly expressed in endothelial cells, cardiac myocytes *(Ballligand et al. (1993))* and cardiac conduction tissue *(Schulz et al. (1991)).*

For the activation of eNOS, Ca^{2+}/Calmodulin complex formation is crucial *(Bredt et al. (1990)*). The complex is formed due to raised concentration of intracellular Ca^{2+} released from intracellular stores, caused by G-proteins stimulation by various substances such as acetylcholine, bradykinin, serotonine or vasopresin. The NO production lasts until the Ca^{2+} level drops, allowing the eNOS to maintain the vascular

tone *(Dinerman et al. (1993))*. Apart from Ca^{2+}-dependency, eNOS activity is regulated by its association with the plasmalemmal scaffolding protein caveolin-1 *(Garcia-Cardena et al. (1996); Shaul et al. (1996))*, post-translational modification *via* phosphorylation by protein kinases and an interaction with the molecular chaperone heat shock protein 90 (Hsp90) *(Garcia-Cardena et al. (1998); Boo et al. (2003); Wei et al. (2005))*. For the final biological activity of NOS, subcellular localization of eNOS is of great importance, if not the most. The eNOS trafficking within the cell is controlled by posttranslational modifications - palmitoylation and myristoylation *(Prabhakar et al. (1998); Yeh et al. (1992))* and also by two interacting proteins - NOSIP ('eNOS interacting protein') and NOSTRIN ('eNOS traffic inducer') that specifically modulate the caveolin/eNOS interaction *(Dedio et al. (2001); Zimmermann et al. (2002))* (Fig. 6).

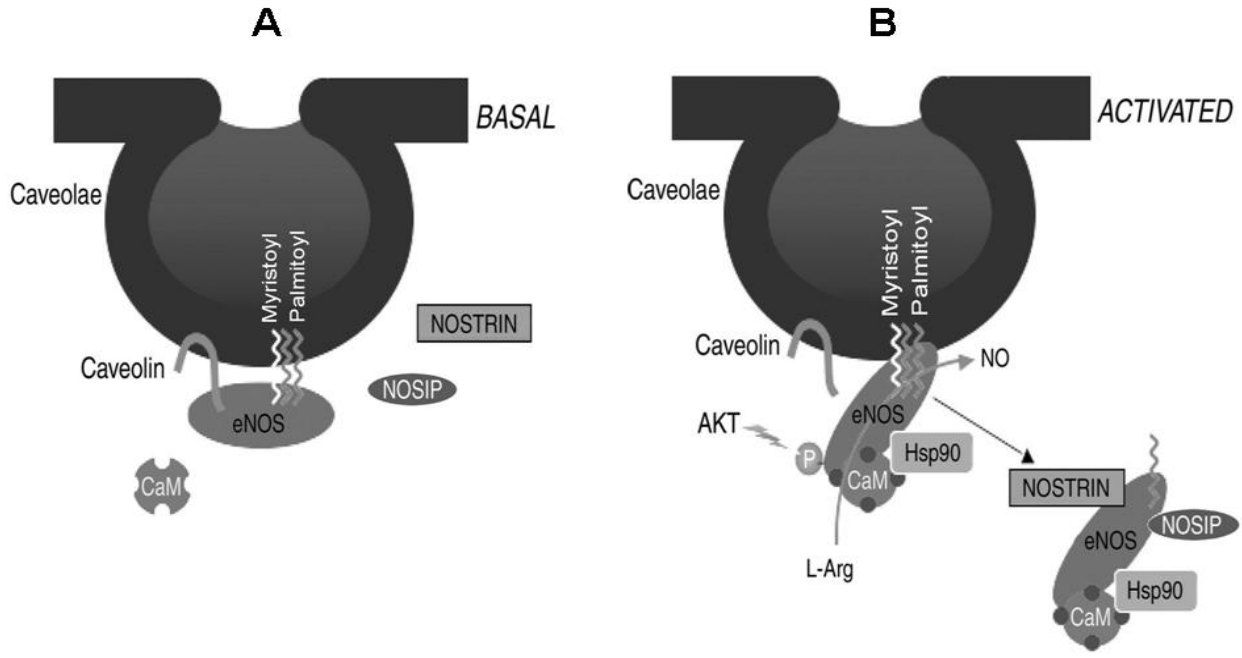

Figure 6. The regulation of eNOS in caveolae. Myristoylation and palmitoylation of eNOS target it to the plasma membrane of caveolae. (A) - Interaction with caveolin inhibits eNOS activity, whereas stimuli such as shear stress promote recruitment of heat shock protein (Hsp) 90 and Ca^{2+}/CaM to eNOS, increasing its activity. (B) - Activated Protein kinase Akt activates eNOS. eNOS interacting protein (NOSIP) and eNOS traffic inducer (NOSTRIN) participate in translocation of eNOS away from the caveolae to intracellular targets, diminishing eNOS activity. (Adapted from Kone et al. (2003))

Produced NO diffuses through the cell membrane into neighboring cells, targeting the function mediator guanylyl cyclase, and ultimately via cGMP activation of a variety of downstream effectors, including cGMP-dependent protein kinase, cGMP-

modulated ion channels, and phosphodiesterases *(Murad et al. (1987)),* causing vascular smooth muscle relaxation *(Ignarro et al. (1990); Ignarro et al. (1986))*. Beyond the vasodilatory effect, NO generated by the endothelial isoform· has a number of other beneficial roles in the vessel wall, including inhibition of vascular smooth muscle proliferation *(Garg et al. (1989))*, reduction of platelet aggregation *(Alheid et al. (1987))*, reduction in expression of adhesion molecules *(Kubes et al. (1991)),* inhibition of lipid oxidation, and regulation of apoptosis *(Chung et al. (2001)).*

Inducible NOS

The immunologic (or inducible) NOS isoform - iNOS (or NOS2) - is the only isoform that is not constitutively expressed. It's responsible for the NO production in macrophages. The human iNOS gene was mapped to chromosome 17 and the protein mass is of 130 kDa *(Chartrain et al. (1994))***.** iNOS is present primarily in the various immunological cells, such as macrophages *(Marletta et al. (1988))* or neutrophils (*Yui et al. (1991))* and is activated by stimulation by bacterial endotoxin, cytokines or tumor necrosis factor *(Stuehr et al. (1987); Ding et al. (1988))*. The regulation of its activity is primarily at the transcription level and it's the only isoform that is inhibited by glucocorticoids *(Radomski et al. (1990))*. In contrast to the other two isoforms, calmodulin is tightly bound to the protein, therefore the changes in Ca^{2+} concentration do not affect the activity. iNOS is also not depending on the cofactors BH_4 and NADPH *(Cho et al. (1992))*.

The induction of iNOS activity is much slower comparing the eNOS and nNOS; it takes several hours from the immunological stimulation to the NO production. On the other hand, the amount of generated NO is far greater *(Welch et al. (1994))* and the protein remains in the cell for several other days *(Xie et al. (1992))*. To obtain cytotoxic levels of NO, concurrence of more signals is required. The exact mechanism of the NO-mediated immunological response is not cleared yet, but its contribution to nonspecific immunity is suggested *(Rodeberg et al. (1995))*. The immunologically induced overproduction of NO and the subsequent vasodilatation are suspected to be responsible for NO's pathophysiological role during sepsis and hypoxia.

Neuronal NOS

The first NOS isoform described to be cloned and sequenced was the neuronal one (nNOS), therefore named NOS1. The human *nNOS* gene has been mapped to chromosome 12 *(Kishimoto et al. (1992))*, and the protein is the largest among NOSs with the molecular mass of 165 kDa. nNOS is present in neurons in the brain and at neuromuscular junctions, and is involved in neurotransmission, memory formation and plays a role in the pathology of central nervous system. It is also present in other tissues like myocard (*Xu et al. (1996))*, skeletal muscle, lung epithelia, and kidney *(Bredt et al. (1991); Mungrue et al. (2004); Papapetropoulos et al. (1999)).*

nNOS is constitutively expressed and is activated by glutamate acting on N-methyl-D-aspartate (NMDA) receptors, which induce the increase of intracellular calcium concentration. The N-terminus of nNOS contains an approximately 100 aminoacids long PSD/Disc-large/ZO-1 homologous (PDZ) domain, that anchors the protein to discrete subcellular membrane regions through protein-protein interactions with cytosceletal elements such as synaptic densities in the nervous system and syntrophin-dystrophin complex in the muscle *(Brenman et al. (1996))*.

The prolonged NMDA receptor activation resulting in excessive increase of calcium is associated with neuron degeneration *(Garthwaite et al. (1989))*, most likely by enhancing NO that interacts with superoxide, subsequently performing its nitrosative reactions *(Lipton et al. (1993))*. This is again an example of ambivalent character of both beneficial and destructive actions of NO, determined by the tempospacial distribution and concentration of NO.

Nitrite and NO

Nitrite is known to have positive effect on the tissue subjected to low oxygen concentrations during hypoxia or anoxia *(Duranski et al. (2005); Webb et al. (2004); Tiravanti et al. (2004))*.

The NO^{2-} anions are unstable intermediates of oxidative transformation of NO to stable metabolite nitrate *(Kelm (1999))*. It's concentration in the vascular system is associated with eNOS activity *(Gladwin et al. (2000); Demoncheaux et al. (2002);*

Kleinbongard et al. (2003); Lauer et al. (2001)). Under normoxic conditions is the nitrite level varying in different tissues from 0.1-1.0 uM in plasma up to 20 uM in vascular tissue *(Bryan et al. (2004); Rodriguez et al. (2003))*. Therefore, nitrite could be viewed as the largest pool of NO in the organism.

Under normal oxygen pressure, there is no evidence of nitrite vasodilatory effects *(Laue et al. (2001))*. The situation changes with the decrease of oxygen tension, suggesting the physiological importance of nitrite for the vasodilatation under these conditions *(Demoncheaux et al. (2002); Cosby et al. (2003))*. The beneficial effect on nitrite is acknowledged to the reduction of nitrite back to NO. There are several ways leading from NO^{2-} to NO. The uncatalyzed reduction is very slow except for extreme acidic conditions present in stomach or ischemic tissue *(McKnight et al. (1997); Tsuchiya et al. (2005); Zweier et al. (1995); Dennis et al. (1991))*. Although prokaryotic nitrite reductases are known, there is no such a dedicated enzyme known in mammals. Instead of a specialized enzyme, a number of mammalian enzymes shows nitrite reductase activity besides their usual function, such as glutathione-S-transferase *(Hill et al. (1992*)), xanthine oxidoreductase (XOR) *(Millar et al. (1998); Godber et al. (2000); Li. et al. (2003)),* deoxyhemoglobin *(Duranski et al. (2005); Webb et al. (2004); Doyle et al. (1981); Luchsinger et al. (2005))* or cytochrome P450 *(Delaforge et al. (1993))*. The mitochondria were also proved to have the nitrite reductase activity, involving cytochrome c oxidase, ubiquinol and complex III *(Castello et al. (2006); Kozlov et al. (1999))*.

Importantly, it was shown that the eNOS, but not nNOS and iNOS, is not only able to produce NO from L-arg together with the oxygen consumption, but is also capable of nitrite reduction to NO under low oxygen conditions, when the conventional arginine pathway is blocked, as was reported for both *in vitro* and *in vivo* experiments *(Gautier et al. (2006); Mikula et al. (2008))*. An anoxic pathway of NO production from nitrite reduction by eNOS was proposed.

Bacterial NOS-like Proteins

The NOS coding genes are extended through the whole animal kingdom and the nitric oxide synthetic activity was observed in the absolute majority of eukaryotes.

Corresponding NOSs were found very similar to the mammalian ones *(Davies et al. (2000)).*

In the bacteria, the situation is more complicated. It is well known, that NO is created as an intermediate of the nitrate or nitrite reduction *(Zumft et al. (1997))*. On top of that, some biochemical studies show that several bacteria, such as *Lactobacillus fermentum, Helicobacter pilori, Neurospora crassa* or *Rhodococcus sp* R312, are capable of L-Arg conversion to citrulin with contemporaneous NO production *(Morita et al. (1997); Stachura et al. (1996); Ninnemann et al. (1996); Sari et al. (1998))*. The bacterial enzyme, responsible for this reaction, was identified in *Nocardia* sp. and named NOCNOS. It occurs as a homodimer, shows activity dependent on NADPH, Ca2+, FAD, FMN and BH_4, and is inhibited by common mammalian NOS inhibitors with close analogy to nNOS *(Chen et al. (1995))*. The purification of an NOS-like protein from Staphylococcus aureus was also described. The majority of cofactors needed for obtaining maximal activity of this protein were the same or very similar to the mammalian NOSs cofactors, nevertheless it exists as a heterodimer *(Hong et al. (2003)).* At the same time, the genome sequencing of various bacteria showed the presence of similar genes in number of other prokaryotes, for example *Deinococcus radiodurans, Bacillus subtilis, Bacillus halodurans, Bacillus anthracis , Staphylococcus aureus, Geobacillus stearothermophilus* etc. *(Zemojtel et al. (2003))*. These suspected proteins were annotated as close homologs of oxygenase domain of mammalian NOSs. Some of these proteins (deiNOS from *D. radiodurans*, bsNOS from *B. subtilis*, saNOS from *S. aureus*, gsNOS from *G. stearothermophilus* and baNOS from *B. anthracis*) were subsequently expressed in *E. coli*, the corresponding proteins were purified and characterized *(Adak et al. (2002); Bird et al. (2002); Midha et al. (2005); Gauthier et al. (2006)).* The crystal structure of bsNOS and saNOS was solved, and it was shown that both proteins are in fact closely related to the oxygenase domain dimer of murine iNOS *(Pant et al. (2002)).*

There are several important components of mammalian NOSs missing in the bacterial analogs - whole C-terminal reductase domain, PDZ-containing N-terminal tail and the cysteines needed for the formation of the zinc tetrathiolate. Sequence corresponding to the pterin binding domain of mammalian NOSs is also present just partially in bsNOS and saNOS. If existing, the pterin binding site is more exposed to the solvent in prokaryota because of lacking several amino acid residues protecting the BH_4- binding site in mammalian proteins, as was shown by X-ray crystal structures.

Nevertheless, the bacterial proteins exist as dimers in solution, therefore an alternative interaction stabilizing the dimer structure is assumed *(Bird et al. (2002); Pant et al. (2002))*.

Some of these bacterial NOSoxy-like proteins catalyze the oxidation of L-Arg or NOHA to citrulline and NO^{2-} *in vitro (Midha et al. (2005))*. The reconstitutive experiments with mammalian NOSs reductase domain showed the transformation of L-Arg or NOHA to citrulline and nitrogen oxides in the presence of NADPH and oxygen, with lower speed comparing to mammalian NOSs though *(Adak et al. (2002)).* Interestingly, some of the NOS-like protein containing bacteria don't have complete BH_4 biosynthetic pathway, but are capable of tetrahydrofolate (THF) synthesis, thus implying that THF acts as the alternative cofactor for the bacterial proteins. The existence and character of those supposed cofactors and associated reductases is not clear yet and it is objective of further research *(Reece et al. (2009))***.**

NO is generally considered to be toxic for the bacteria *(Kaplan et al. (1996); Nioche et al. (2004))*. Lately it was shown that some bacteria also may use NO for protection against oxidative stress *(Gusarov et al. (2005))*. But the real role of prokaryotic NOS-like proteins and NO itself remains controversial. Up to date the relation between NOS-like proteins expression is still unclear. It has been shown that the protein from bacterium *Streptomyces turgidiscabies* takes part in the nitration reaction that occurs during the biosynthesis of phytotoxin thaxtomycin, which implies its role in pathogenicity of this bacterium *(Kers et al. (2004)).* Further, protein deiNOS from *D. radiodurans* has been described to be associated with unusual tryptophanyl tRNA synthase and the formed complex shows higher activity during NOHA oxidation and regioselective tryptophan nitration *(Buddha et al. (2004)).*

Bacterial NOS analogs turned to be very useful for the study of their mammalian counterparts as well. The structural studies revealed that despite slight differences in the amino acid composition, the conformation of the active site remains very similar to that of mammalian isoforms. Thanks to that the prokaryotic proteins were successfully used to reveal the inner work of the eukaryotic NOSs *(Salard et al. (2006))*. The differences of particular bacterial protein properties caused by differences in the active center structure correlate with the differences between particular mammalian NOSs as well. It led also to the hypothesis of the second NO-binding site in the active center of eNOS *(Gautier, Mikula et al. (2006); Gautier, van Faassen et al. (2006))* or to

surprising differences in the interaction of individual proteins with L-Arg analogs and Fe-ligands *(Salard et al. (2006))*.

Mitochondrial NOS

NO has profound effect on many physiological and pathophysiological processes in mitochondria (Fig. 7), including reactive oxygen species (ROS) formation, energy metabolism and the regulation of oxygen consumption *(Giulivi et al. (2006))*. Reaction with the superoxide anion, also formed in mitochondria, results in the formation of peroxonitrite ($ONOO^-$), an important apoptotic factor *(Vicente et al. (2006))*. Other reactive nitrogen species, such as S-nitrosothiols (RSNOs) or nitroxyls are derived from NO *(Boveris et al. (2002); Ghafourifar et al. (1999); Poderoso et al. (1999); Cadenas et al. (2000); Radi et al. (2002))*. The S-nitrosylation by NO is involved in protein thiol-group protection under oxidative stress and is believed to allow reversible protein function regulation (*Whiteman et al. (2006))*. NO itself interacts with cytochrome oxidase, thus influencing the mitochondrial respiration *(Moncada et al. (2006); Prime et al. (2009))*.

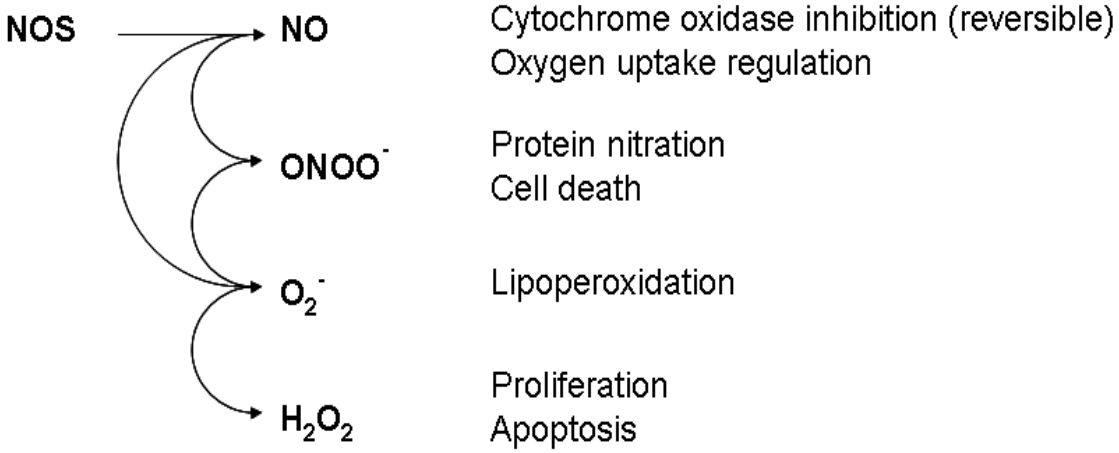

Figure 7. The effects of NO reactions in mitochondria. In aerobic conditions, nitric oxide decays primarily producing peroxynitrite and hydrogen peroxide. Considering the high affinity of NO for cytochrome oxidase, this pathway is important to avoid a sustained inhibition of the mitochondrial transfer chain. Additionally, the mechanism will become a dangerous one, whether or not NO concentration increases in the mitochondrial matrix. The consequence will

be the oxidation of proteins and lipids, mitochondrial damage and alterations in the life cycle, as judged by mitochondrial influence on cell apoptosis. (Adapted from Carreras et al. (2000))

The existence of mitochondrial nitric oxide synthase remains to be a controversial topic. The first reports suggesting the presence of NOS in mitochondria based on anti-NOS antibodies staining mitochondria in couple of immunohistochemical studies *(Bates et al. (1995); Frandsen et al. (1996); Kobzik et al. (1995))* were published in the middle 90's. Since then an intensive search for the mtNOS has been proceeding to this day without final conclusion, which would be accepted in the scientific society.

There are many publications from different groups describing biochemical properties of mtNOS from various tissues *(Ghafourifar et al. (1997); Giulivi et al. (1998); Tatoyan et al. (1998))* or even the activity in the purified mitochondria *(Navarro et al. (2008)*). As well as there are communications identifying mtNOS as eNOS *(Bates et al. (1996); Hotta et al. (1999); Zanella et al. (2004))***,** other that denote iNOS to be the mitochondrial one *(French et al. (2001); Gonzales et al. (2005); Valdez et al. (2004))* and perhaps the largest group that claims that mtNOS is a splice variant of nNOS localized in the inner mitochondrial membrane *(Kanai et al. (2004); Elfering et al. (2002); Dedkova et al. (2006))*. The last theory is also supported by the indirect proof describing absence of NOS activity in nNOS knock-out mice *(Kanai et al. (2001)).* On the other hand, there is a number of works calling a question or straightly refuting the existence of mitochondrial NOS derived from the known three isoforms, considering the above mentioned papers as equivocal and showing evidence of absence of NOS in mitochondria *(Brookes, P.S. (2004); Ghafourifar et al. (2005); Lacza et al. (2009); Venkatakrishnan et al. (2009))*. Additionally, NO can be produced inside mitochondria by other NOS independent pathways *(Kozlov et al. (1999); Basu et al. (2008); Castello et al. (2006))*.

Protein NOA1

In 2003 Crawford group published a paper in Science *(Guo et al. (2003))* identifying a brand new protein in *Arabidopsis thaliana* named AtNOS1 as a potential

nitric oxide synthase. This was based on the sequence homology of AtNOS with a snail protein from *Helix pomatia*, described previously in a solitary paper as a nitric oxide synthase or a NOS-interacting protein *(Huang et al. (1997))*. Crawford group showed reconstitution of AtNOS knock-out plants by NO donors or isolated protein itself, and also measured the NOS activity of isolated AtNOS. Reports on reduced levels of NO in AtNOS depleted plant followed *(Guo et al. (2005); Bright et al. (2006); Zeidler et al. (2004)).*

Interestingly, AtNOS showed no sequence similarity to known NOSs. It is a much shorter protein of 561 aminoacids with the only homology to the circularly permutated GTPase family *(Anand et al. (2006))*. The closest homolog to AtNOS is bacterial protein YqeH from Bacillus subtilis, belonging to a subfamily of cGTPases *(Morimoto et al. (2002))*. Importantly, AtNOS was reported to contain a mitochondrial targeting sequence and its mitochondrial localization was shown *(Guo et al. (2005); Zemojtel et al. (2006))*. Based of these findings, AtNOS was proposed to be a novel putative mitochondrial NOS, not related to the already known nitric oxide synthases.

Later on, several groups including our lab failed to reproduce the originally reported NOS activity and the function of the protein has been questioned. The protein was renamed to NO-associated protein 1 (AtNOA1). It was showed recently that AtNOA1 is not capable of arginine-dependent NOS activity but it's a functional GTPase instead *(Moreau et al. (2008))* and the crystal structure of the plant protein was solved *(Sudhamsu et al. (2008)).*

Independently, the YqeH protein function was uncovered to be involved in bacterial ribosomal assembly *(Anand et al. (2010); Anand et al. (2009))*. The latest work confirmed that the eukaryotic analog of YqeH, the NOA1 presents the same function in higher organism, and with regard to its localization in mitochondria or plastids in plants, it's is essential for proper mitochondrial and chloroplast ribosome assembly and/or translation *(Kolanczyk et al. - submitted to JBC; Liu et al. (2010))*.

Aims of the thesis

The overall aim of this thesis was the structure-functional study of both eukaryotic and prokaryotic representatives of nitric oxide synthases in order to better understand the mechanisms of NO production and regulation. The specific aims are listed below:

- to test the capability of mammalian NOSs to produce NO by the reduction of nitrites under physiological conditions, different availability of oxygen and diverse nitrite concentrations, by various methods, including absorption spectroscopy, electron paramagnetic resonance (EPR) spectroscopy and electrochemical measurements, *in vitro* and *in vivo*. Compare the nitrite reductase activities of distinct NOS isoforms, with special focus on hypoxic and anoxic conditions

- to study the kinetic and dynamic properties of the heme environment of nitric oxide synthase, using bacterial NOS-like proteins, which show high homology with the oxygenase domain of mammalian NOS, as a model system and comparing them with the mammalian NOSs

- to perform a detailed comparative study of the interactions of a series of substrate analogs, inhibitors and iron ligands of mammalian NOSs with the recombinant bacterial NOS-like proteins

- to characterize the newly described protein AtNOS1, proposed to be a putative nitric oxide synthase in plant *Arabidopsis thaliana*. Uncover the role of mammalian orthologs

Results And Discussion

Dynamics of NO rebinding to the heme domain of NO synthase-like proteins from bacterial pathogens

In this paper we studied the kinetic and dynamic properties of heme environment of bacterial NOS-like proteins, using ultrafast transient spectroscopy. The NOS-like proteins (or HNS - heme domain of NO Synthase-like proteins) exhibit high homology with the oxygenase domain of mammalian NOSs. Previously reported structural and spectroscopical studies of NOS-like proteins from *B. subtilis* (BA-NOS) and *S. aureus* (SA-NOS) showed high conformational similarity in the heme neighborhood compared to that of mammalian NOSs *(Pant et al. (2002); Bird et al. (2002); Martasek et al. (2001); Raman (2001)).*

Thus, the bacterial proteins provide us an excellent model system to disclose the inner-workings of mammalian NOS. Recombination of NO ligand after photodissociation is a first-rate tool to probe heme conformations within these bacterial proteins and comparing them with the oxygenase domain of the endothelial NO-synthase (eNOSoxy), with high relevance to mammalian NOS regulation *(Negrerie et al. (1999); Slama-Schwok et al. (2002)).*

We showed that NO rebinding in both SA-NOS and BA-NOS is very similar in the absence of L-Arg and markedly faster compared to eNOSoxy. In the presence of substrate, the BA-NOS NO-rebinding slows down, resembling that of eNOSoxy, while the SA-NOS NO-rebinding is markedly faster.

To test the hypothesis of a second NO-binding site in the proximity of the active center of eNOSoxy, that was raised by Slama-Schwok and coworkers previously *(Slama-Schwok et al. (2002))*, we performed the rebinding kinetics measurements at various temperatures and NO concentrations. The obtained data are fully consistent with different barriers for NO recombination in SA-NOS and BA-NOS and strongly supports the hypothesis of the existence of second NO-binding site.

From the calculations of enthalpies for the energetic barriers result, that the second NO-binding site is present in eNOSoxy as well as in the BA-NOS, in the SA-

NOS is either missing or is isolated, which would explain lack of the effect of NO concentration on the geminate rebinding in SA-NOS.

The effect of NO concentration on the NO rebinding to eNOSoxy disappears in the absence of L-Arg and BH_4. According to this, it was proposed that NO dynamic and escape from eNOS is regulated by the active site H-bonding network connecting between the heme, the substrate, and cofactor. The H-bonding is likely to modulate the energy barriers for NO recombination and modify the heme redox potential. Similar hydrogen bonding network in eNOS and BA-NOS might result in a similar NO rebinding in the presence of the arginine substrate, in contrast with slightly different H-binding network architecture.

Interestingly, in both bacterial NOS-like proteins the lack of arginine resulted in the transition of NO-ferrous complex from hexacoordinated complex to the pentacoordinated state. The same behavior was described previously for nNOS. *(Santolini et al. (2001); Abu-Soud et al. (2000)).*

In this work I was responsible for all the recombinant protein preparations (overexpression, isolation and purification, purity a heme content determination, the confirmation of activity) and further participated in the NO recombination measurements, especially for both the bacterial NOS-like proteins.

Analogies and surprising differences between recombinant nitric oxide synthase-like proteins from Staphylococcus aureus and Bacillus anthracis in their interactions with L-arginine analogs and iron ligands

In this work we performed a detailed comparative study of the interactions of a series of L-arginine (L-arg) analogs, inhibitors and iron ligands of mammalian NOSs with two recombinant NOS-like proteins from bacteria Staphylococcus aureus (SA-NOS) and Bacillus anthracis (BA-NOS) by the methods of UV–visible spectroscopy.

Both bacterial proteins were overexpressed in *E. coli* and purified in the absence of added pterin and L-Arg following the protocol, described before *(Chartier et al. (2004); Midha et al. (2005); Gauthier et al. (2006); Martasek et al. (2001))*. The heme content of the prepared proteins were determined from their [FeII–CO]–[FeII] difference spectrum using an extinction coefficient $De_{444–470}nm$ = 76 $mM^{-1}.cm^{-1}$ (*Adak et al. (2002); Stuehr et al. (1992))* and the purity of the samples were judged by SDS-PAGE to be more than 95%.

The results confirmed that both SA- and BA-NOS, in their native ferric state, as well as their complexes with various substrates and ligands, behave as heme-thiolate proteins and exhibit spectral properties similar to mammalian NOSs or cytochromes P450. Both bacterial proteins react with NO forming heme-FeIII-NO and heme-FeII-NO complexes. The heme-FeIII-NO complex is stable for SA-NOS and not for BA-NOS, whereas both ferrous complexes are unstable at room temperature.

Both proteins bind imidazole (Im) and the affinity for the substituted Im decrease with growing size of the derivates. The two bacterial proteins, as mammalian NOSs *(Lefevre-Groboillot et al. (2005))*, exhibit a better affinity for L-arginine than for Homo-L-arginine, and fail to bind Nor-L-arginine. In contrast to the mammalian NOSs, the two bacterial NOSs exhibit a better affinity for competitive inhibitors of mammalian NOSs NO_2-L-arginine (NO_2-L-Arg), N-methyl-L-arginine (NMMA), and N- iminoethyl-L-ornithine (NIO) than for L-arginine itself whereas L-NOHA tightly binds to both proteins as well as to mammalian NOSs *(Kerwin et al. (1995); Furfine et al. (1993)).*

On top of this, we showed that SA-NOS significantly differs from BA-NOS at the level of several important properties. In the native state, saNOS shows absorbance maxima at 415, 535 and 641 nm, characteristic for the hexacoordinated low-spin ferric state, whereas native BA-NOS absorbance maxima are at 395, 518 and 651 nm, that

indicates pentacoordinated high-spin form identical to those of iNOS oxygenase domain containing BH_4 an L-Arg*. (Ghosh et al. (1997); Mayer et al. (1997); Presta et al. (1998); Chabin et al. (1996))*.

The proteins differ in their respond to the addition of BH_4 or its analogs. The affinity of substrate L-Arg for SA-NOS increases after the addition of pterin, but there is no effect in the BA-NOS. There is also important difference in binding of various ligands, the SA-NOS protein, in contrast to BA-NOS, binds relatively large ligands such as nitrosoalkanes and tert-butylisocyanide.

Thus, SA-NOS exhibits properties very similar to those of the oxygenase domain of inducible NOS (iNOSoxy) not containing H4B, as expected for a NOS-like protein that does not contain BH_4. By contrast, the properties of baNOS which look like those of BH_4-containing iNOSoxy are unexpected for a NOS-like protein not containing BH_4. Our results would suggest that BA-NOS mainly exists as a tight dimer whereas native SA-NOS would mainly exist as a loose dimer that can accommodate a wider range of ligands. Interestingly, a loose dimer structure has been recently characterized crystallographically for the NOS-like recombinant protein from B. subtilis *(Pant et al. (2005)).*

The present study also led to interesting data for the design of strong specific inhibitors of those bacterial NOSoxy-like proteins. NO_2-L-arg and SEITU exhibit a high affinity for BA-NOS. These compounds could be interesting tools for future studies of BA-NOS.

My contribution to this work was the overexpression, isolation and purification of recombinant SA-NOS and BA-NOS proteins used for the spectroscopic measurements and determination of their purity and heme content.

Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia

Some mammalian proteins such as glutathione-S-transferases *(Hill et al. (1992))*, xanthine oxidoreductase (XOR) *(Millar et al. (1998)*), deoxy-hemoglobin *(Doyle et al. (1981); Duranski et al. (2005); Luchsinger et al. (2005))*, and cytochrome P-450 enzymes shows some nitrite reducing capacity, therefore in this communication we scrutinized NOS for a possible nitrite reductase capability by absorption spectroscopy, electron paramagnetic resonance (EPR) spectroscopy and electrochemical measurements.

We showed that purified endothelial NOS reduces nitrite ions to NO under anoxic conditions *in vitro*. The release of NO from nitrite at physiological pH was confirmed using three independent methods. The nitrite reduction was shown to be performed by the oxygenase domain of the protein, independently on the occurrence of pterin cofactor - tetrahydrobiopterin.

The NO production at the physiological pH under hypoxia by nitrite reduction increases by six fold with respect to the regular arginine pathway, which is largely blocked under low oxygen tension conditions. Therefore, basal levels of NO release could be sustained by anoxic nitrite reduction. The reaction suggests a new pathway for fast NO delivery under hypoxia, precisely when the vasodilatory properties of nitric oxide are most needed. The magnitude of the NO release suggests that the nitrite reductase activity of eNOS has relevance for a fast NO delivery in tissues under acute hypoxia and the fast burst of nitrite- derived NO could assist in maintaining vasodilatation and minimal blood flow under low oxygen tension.

I participated on this work by the preparation of recombinant protein samples, for the experiments, and the measurements of NO production using electrochemical detection with NO-electrode.

Isoform-specific differences in the nitrite reductase activity of nitric oxide synthases under hypoxia

In the present study, we compared the nitrite reductase activities of three mammalian NOS isoforms under anoxic conditions, using absorption spectroscopy, electrochemical detection and chemiluminiscence. Since several other hemoproteins such as hemoglobin and cytochrome *c* are also known to act as nitrite reductases under hypoxia *(Gladwin et al. (2000); Duranski et al. (2005); Crawford et al. (2006); Rassaf et al. (2007); Cosby et al. (2003); Castello et al. (2006))*, we also investigated the role of the heme domains in each of the isoenzymes in the nitrite reduction. Since the three NOS isoforms share high homology, we could adequately test requirements of a heme and/or flavins for nitrite reduction. Additionally, we compared the effect of anoxia at physiological (15 uM) and supraphysiological (500 uM) nitrite concentrations or 10 uM nitrate.

NO production from nitrite (at physiological level - 15 uM) under anoxic conditions was specific only to the endothelial NOS, whether nNOS and iNOS showed no nitrite reducing activity. According to our data we proposed that structural and, by inference, dynamic differences between nNOS and eNOS in the distal heme site may be the reason for eNOS being the only isoform capable of converting nitrite into NO at physiological pH. High nitrite concentrations largely decreased the probability of NO release from the reduced heme of eNOS, as shown by ultrafast optical spectroscopy. Further, we tested whether isolated NOS domains are capable of nitrite reduction. The reductase NOS domains were unable to recycle nitrite to NO under anoxia, whilst oxygenase domains of both eNOS and nNOS released NO, emphasizing the importance of heme in nitrite reduction and suggesting that reductase domain modulates the nitrite reducing activity of NOS.

To test NO formation from nitrite by the NOS in vivo, we performed cellular study in human dermal microvascular endothelial cells (HDMECs) using 10uM nitrite. The experiments were under strict control of oxygen tension to obtain condition where the aerobic arginine-dependent NO production is blocked, which means that the [O_2] was kept below the K_m for oxygen. The chemiluminiscence measurement showed that hypoxic (2 ppm O_2) NO formation was enhanced approx. 3.6-fold compared to normoxia (22 ppm O_2), and under completely anoxic conditions (argon atmosphere)

8.3-fold higher actually. The NO formation was inhibited by addition of NOS inhibitor L-*N*-methylarginine (L-NMA), but not by nNOS specific inhibitor 3-bromo-7-nitroindazole. We also used the xanthine oxidas (XO) inhibitor oxypurinol to exclude XO to be the source of NO.

Both in vitro and in vivo results show the eNOS to be the only isoform to have a nitrite reductase activity under all oxygen level from normoxia to complete anoxia at physiological concentrations of nitrite. The magnitude of NO release by the endothelial under hypoxia cells suggest that the endothelium could provide an appropriate response to acute episodic ischemia and may explain the observed eNOS-expression-specific protective effect as a short-term response in animal models of acute hypoxia.

My participation on this work consisted of the recombinant protein preparations (overexpression, isolation and purification, purity a heme content determination, the confirmation of activity) and I was concerned in the *in vitro* NO production measurements and NO rebinding assays.

Mammalian mitochondrial nitric oxide synthase: Characterization of a novel candidate

This work was triggered by the recent findings of protein AtNOS1 from *A. thaliana*, described by Crawford and coauthors *(Guo et al. (2003))* as a putative nitric oxide synthase in plants. AtNOS1 was reported to be associated with arginine dependent NO production in *Arabidopsis*, and interestingly, it shows no homology to the already known animal NO-synthases. We focused in our work on the functional characterization of mammalian ortholog (murine AtNOS1) of the plant AtNOS1.

We characterized the subcellular localization of the mammalian AtNOS1 to be functional in the context of mitochondria using cellular fractionation, immunolabeling of constructed fusion proteins and by immune-gold electron microscopy. It was clearly shown that the protein localizes to the inner-mitochondria compartment, with high probability that the inner mitochondrial membrane is the primary site of mAtNOS1 localization.

On top of that, we confirmed the N-terminal mitochondrial targeting sequence present in mAtNOS1 gene, predicted by the sequence analysis, using N-terminus deletion mutants of mAtNOS1 protein. Using fusion protein of N-terminal 1-60 aminoacids from mAtNOS1 with enhanced green fluorescent protein (EGFP) we demonstrated this region to contain a mitochondrial targeting signal that is necessary and sufficient for import into mitochondria.

The analysis of the embryonic expression pattern of mAtNOS1 gene revealed a weak, but widespread expression. In situ hybridization data suggest, that mAtNOS1 may play a role in development of neural, hematopoietic and bone organ systems. The Northern blotting using RNA from adult mouse organs further revealed expression of the *mAtNOS1* gene in the organs associated with high mitochondria content, like testes, heart, liver, brain and thymus. Together, the expression data suggested that the candidate for mammalian mitochondrial nitric oxide synthase contributes to multiple physiological processes during embryogenesis, which may include roles in liver hematopoesis and bone development.

In this work I was concerned in the preparation of the fusion genes, preliminary trials in the recombinant mice and human AtNOS1 ortholog proteins preparation and NO activity measurements, and identification of their mitochondrial interaction partners.

Corrigendum to ‘‘Mammalian mitochondrial nitric oxide synthase: Characterization of a novel candidate’’

In the corrigendum to our previous report we updated our paper according to the latest findings in the field.

More working groups, including ours, failed to detect any NO synthetic activity in purified AtNOS1 protein from various species (namely mice and human in our laboratory). Also Crawford group, originally reporting AtNOS1 as a putative NOS, claimed to have no longer data support of the arginine-dependent NOS activity of AtNOS1. The protein was renamed to AtNOA1 (Nitric Oxide Associated 1).

The bioinformatics studies revealed the presence of circularly permuted GTP-binding domain in the *AtNOA1* gene, and homology to the bacterial YqeH protein, a confirmed GTP-binding protein suggested to be required for proper 70S ribosome formation and, in particular, 30S subunit assembly/stability in *B. subtilis (Uicker et al. (2007))*. Based on learned information, we have hypothesized an involvement of eukaryotic orthologs of AtNOA1 in mitochondrial ribosome biogenesis and/or processes of translation.

In this work I was concerned in the preparation of the fusion genes, preparation of recombinant mice and human AtNOS1 orthologs and NO activity measurements and identification of their mitochondrial interaction partners.

Plant nitric oxide synthase: a never-ending story?

This short paper turned out to be one of the most important publications regarding AtNOA1 and widely NO signaling in plants. Over 100 citations in 4 years prove its significance.

Despite the effort that our group and several other groups made to confirm the NO-synthetic activity of recombinant AtNOS1 orthologs from various species (mouse, human, rice, maize), we failed to detect any NO production using [3H]-arginine and Greiss reagent-based NOS assays. Moreover, AtNOS was identified as a member of a novel evolutionary conserved GTP-binding protein family *(Zemojtel et al. (2004))*, with members in organisms ranging from bacterium to human. According to these findings, we raised a critical question regarding the function of AtNOS1.

We proposed several hypotheses, including AtNOS1 being only a subunit of a larger NO-producing complex or its localization upstream of NO production. The most credible hypothesis arose from recent reports on the bacterial protein YqeH, identified by us as a Bacillus subtilis ortholog of AtNOS1. YqeH was shown to bind GTP and GDP *(Morimoto et al. (2002))* and assigned to the a YlqF/YawG protein family containing a circularly permuted GTPase domain, that includes members involved in ribosomal biogenesis and/or the translation process *(Leipe et al. (2002); Himeno et al. (2004)).* Based on all these data we proposed that AtNOS1 is a GTPase involved in mitochondrial ribosome biogenesis and/or processes of translation. In this scenario, *AtNOS1* deletion would lead to defects in mitochondrial biogenesis resulting in an observed decrease in NO production.

My contribution to this report were the unsuccessful trials to confirm the NOS activity of AtNOS orthologs, starting from cloning the genes, overexpression in the *E. coli*, isolation and purification of recombinant proteins and ending with NOS activity measurement.

NOA1 is essential GTPase required for mitochondrial protein synthesis and apoptosis

It has been shown recently, that NOA1 is an evolutionarily conserved GTP binding protein which localizes predominantly to mitochondria in mammalian cell. Based on bioinformatic analysis we predicted in our previous works *(Zemojtel, Fröhlich et al. (2006); Zemojtel, Kolanczyk et al. (2006))* its possible involvement in the ribosomal biogenesis.

In this communication we analyzed NOA1 function through generation of the knock-out mice and in the *in-vitro* assays with the purified to homogeneity protein. *NOA1* deficient mice exhibit mid-gestation lethality associated with severe developmental defect of the embryo proper as well as severe defects of the placenta with a reduction of all three trophoblast layers including the trophoblast giant cell layer (TGC). The transmission electron microscopy (TEM) of *Noa1-/-* embryos revealed abnormal mitochondria with characteristically swollen cristae, but no abnormalities in other organelles.

The experimental data obtained from polarographic, biochemical and spectroscopic assays together with the blue-native electrophoresis showed show impaired mitochondrial protein synthesis and a global defect in oxidative phosphorylation (OXPHOS) in the primary embryonic fibroblasts isolated from NOA1 knock-out embryos. Additionally, *Noa1-/-* cells are impaired in the staurosporine induced apoptosis.

The analysis of mitochondrial ribosomes by the sucrose gradient centrifugation and Western blotting showed that the mitochondrial large ribosomal subunit from *Noa1-/-* cells sediments anomalously suggesting defect in assembly. In line with that, *in vitro* experiments revealed that purified mammalian NOA1 displayed, like its bacterial homolog YqeH, GTPase activity. This activity was stimulated by rRNAs of the small and large ribosomal subunits from both mammalian mitochondria and *E. coli*.

Taken together, our data show that NOA1 is an essential component of the mitoribosomal assembly machinery with a conserved function during evolution of the eukaryotic cells, therefore is required for mitochondrial protein synthesis, and such basal mitochondrial functions as ATP synthesis and apoptosis.

I contributed to this work with the preparation of the human NOA1 protein (cloning of the gene, overexpression of the recombinant protein in *E. coli*, isolation and

purification), that were used for the ribosomal reconstitution assays and GTPase activity measurements.

Conclusions

The presented work significantly contributed to the understanding of NO production by nitric oxide synthase proteins. It widened the knowledge of the mechanisms of control and regulation of the NO production on submolecular level, described new characteristics of distinct NOSs and NOS-like proteins and compared them with each other, confirmed a new pathway for NO generation under specific conditions and displaced the NO-synthetic activity in the proposed novel NOS protein (AtNOS1) by the evidence of different physiological function.

- A detailed comparative study, by UV–visible spectroscopy, of the interactions of a series of usual substrates, inhibitors, and iron ligands of mammalian NOSs with recombinant bacterial NOS-like proteins SA-NOS and BA-NOS from S. aureus and B. anthracis was done. The results confirmed their behavior as heme-thiolate proteins with spectral properties similar to mammalian NOSs, but also revealed differences in the interactions of these two proteins with some L-Arg-derived compounds and iron ligands, and showed that BA-NOS exhibits unusual properties compared to BH_4-free mammalian iNOS.

- The comparative study, by ultrafast transient spectroscopy, of the NO-dynamics in the active center of the bacterial NOS-like proteins and eNOS and its tuning by the heme environment led to the proposition of a second NO-binding site in the catalytic site of some of the NOSs, and a unravel the regulation of NO dynamic and escape from by the active site H-bonding network connecting between the heme, the substrate, and cofactor.

- It was demonstrated, *in vitro* and also *in vivo* in endothelial cells, that only eNOS, but not nNOS and iNOS, is capable of reduction of nitrite anions to NO at physiological pH and nitrite levels, under oxygen levels from normoxia to complete anoxic conditions. A new, arginine-independent pathway for fast NO-delivery under hypoxia and anoxia was introduced.

A new protein - AtNOS1 or NOA1 after renaming - was characterized on functional level, and although it turned out to has completely different function than initially claimed nitric oxide synthetic activity, or at least association with NO

production, the reported mitochondrial localization and its role in mitoribosomal assembly open a brand new field for the further research.

The results presented in this thesis have important impact on the further research not only at the NO field. The better understanding of the exact mechanisms of NO synthesis in the active center of NOSs and subsequent release of NO, together with detailed information about other ligand interactions could facilitate the design of potent, selective and specific modulators (both inhibitors and activators) of mammalian NOSs, but also of the NOS-like proteins of bacterial pathogens, thus influencing their pathogenicity.

The elucidation of NOS-derived NO biosynthesis under lowered oxygen by nitrite reduction is important for the elucidation of the physiological processes in organisms during acute ischemic conditions and could potentially lead to the new approaches in their treatment, helping to overcome the acute ischemic damage.

And finally, the description of the proper biological function of a new protein NOA1, and its importance, provides background for further research with far-reaching possibilities of applications in the future.

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Supplements

Original communications