Charles University in Prague

1st Faculty of Medicine

PhD thesis summary

Eukaryotic and Prokaryotic Nitric Oxide Synthases – Structure-Function Studies

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Prague 2010

Postgraduate studies in Biomedicine

Charles University in Prague
and Academy of Sciences of the Czech Republic

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PhD thesis summary was sent:

Defense of the thesis:

The full text of the PhD thesis is available at the dean's office of the 1st Faculty of Medicine, Charles University in Prague

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Abstrakt in Czech

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, neboli také imunologická (iNOS). Jednotlivé izoformy mají významnou podobnost sekvencí (až 47-55%), a jsou aktivní pouze ve formě homodimeru. Každý monomer se skládá se ze dvou rozdílných, katalyticky aktivních domén. N-terminální oxygenázová doména (NOSoxy) obsahuje vazebná místa pro prostetickou skupinu hemu, kofaktor tetrahydrobiopterin (BH_4) a substrát L-arginin, zatímco C-terminální reduktázová doména (NOSred) obsahuje vazebná místa pro flavinadenindinukleotid (FAD), flavinmononukleotid (FMN), a kosubstrát nikotinamidadenindinukleotidfosfát (NADPH).

Některé bakterie nesou geny kódující proteiny homologní s oxygenázovou doménou savčích NOS a vykazující NO-syntázovou aktivitu *in vitro*. Díky svým unikátním vlastnostem slouží jako vynikající modelový systém pro studium aktivního centra savčích NOS. Vzhledem ke skutečnosti, že NO je pleiotropický regulátor buněčných funkcí, předpokládá se, že NO tvořený patogenními organismy jako je *B. anthracis* nebo *S. aureus* by mohl hrát kritickou roli v patofyziologickém průběhu infekce.

Komparativní spektroskopická 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 pomocí techniky "ultrafast transient spectroscopy" 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. Redukce nitritů probíhá v organismu buď přímo v extrémně kyselém prostředí, např. v žaludku, nebo je katalyzována některými enzymy s jinou primární fyziologickou funkcí.

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 navržena a následně prokázána jeho funkce v asemblaci mitochondriálního ribozomu a tím i syntéze mitochondriálních proteinů a základních mitochondriálních funkcích včetně syntézy ATP a apoptózy.

Abstract in English

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). All three isoforms share a sequence homology of 47-55% and are active only in the homodimeric form. Each monomer consists of two different catalytically active domains. The N-terminal oxygenase domain (NOSoxy) contains binding sites for heme prosthetic group, cofactor tetrahydrobiopterin (BH_4) and substrate L-arginine. The C-terminal reductase domain (NOSred) contains binding sites for *flavin adenine dinucleotide (FAD),* Flavin mononucleotide (FMN) and cosubstrate nicotinamide adenine dinucleotide phosphate (NADPH).

Some bacteria harbor genes coding for proteins homologous to the mammalian NOS oxygenase domain and showing NO-producing activity *in vitro.* Thanks to unique properties they provide us with an excellent model system for the study of the active center of mammalian NOSs. Due to the fact that NO is a pleiotropic regulator of cell functions, it is supposed that NO generated by pathologic organisms such as *B. anthracis* and *S. aureus* could play a critical role in the pathophysiological processes during the infection.

The comparative spectroscopic 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 by the ultrafast transient spectroscopy, 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. Nitrite reduction proceeds either directly under extremely acid conditions, e.g. in stomach, or is catalyzed by enzymes with different primary physiological function.

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 NOS, showing no homology to the known NOSs.

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 proposed and subsequently proved to take place in the mitochondrial ribosome assembly, mitochondrial protein synthesis, and such basal mitochondrial functions as ATP synthesis and apoptosis.

Review of the Literature

Nitric oxide

Nitric oxide (NO) 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)).* 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. NO is very susceptible to reaction with iron, thus forming a complex in the heme group. Heme is a component of many metalloproteins, called hemoproteins, e.g. cytochrome P450, catalases, nitric oxide synthases (NOS) and soluble guanylate cyclase (sGC) *(Wink et al. (1993)).* The activation of sGC by interaction with NO and consecutive cGMP production is considered to be the main NO-signaling pathway *(Koesling et al. (2000)).* 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)).*

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 active only in the homodimeric form, the monomeric form is inactive. The molecule (subunit) of NOS consists of the N-terminal oxygenase domain, C-terminal reductase domain and calmodulin-

binding side in-between. The reductase domain contains a binding site for the electron donor NADPH as well as the biding sites for two flavin cofactors – FAD and FMN. The domain structure is very similar to NADPH-dependent cytochrome P450 oxidoreductase (CYPOR) *(Bredt et al. (1991)).*The oxygenase, or also heme domain, is at the N-terminal half of the NOS subunit. It contains a heme (iron protoporphyrin IX) as a prosthetic group in the active site, and the binding sites for pterin (BH_4) and for the substrate L-Arg *(McMillan et al. (1993)).* 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. The electrons are transported from NADPH through flavin cofactors FAD and FMN to the heme active center of the oxygenase domain of the opposite dimer subunit *(Griffith et al. (1995)).* Another important structural element present in NOS is the zinc tetrathiolate cluster, which contributes to the stabilization of the dimer *(Ludwig and Marletta (1999)).*

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

The direct product of the catalysis is not free NO, but a ferric heme-NO complex *(Negrerie et al. (1999)).* The mechanism and kinetics of the NO release from this complex is indeed 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. An important factor affecting fundamentally the NO production is the external NO, which is already present in solution. The effect of NO solution concentration differs significantly among NOS isoforms *(Abu-Soud et al. (2001)).*

Mammalian NOS Isoforms

In mammals, there are at least three different isoforms of NOS described. There were originally identified and named according to the tissue 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 different gene. According to the enzyme commission nomenclature, the proteins share the same EC number 1.14.13.39.

Due to 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 *(Forstermann et al. (1991))*. The inducible isoform iNOS has calmodulin (CaM) bound permanently as an additional subunit and hence is not regulated by CaM binding, but is under transcriptional control instead *(Cho et al. (1992))*.

The intracellular localization of distinct isoforms determines the accessibility to the extracellular stimuli and to the efficiency of NO production *(Nedvetsky et al. (2002))*. 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))*.

eNOS 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 and cardiac conduction tissue *(Ballligand et al. (1993))*.

eNOS-derived 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, causing vascular smooth muscle relaxation *(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 is the only isoform that is not constitutively expressed. It's responsible for the NO production in macrophages. iNOS is present primarily in the various immunological cells and is activated by stimulation by bacterial endotoxin, cytokines or tumor necrosis factor *(Stuehr et al. (1987))*. The regulation of its activity is primarily at the transcription level *(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 does not affect the activity. iNOS is also not depending on the cofactors BH_4 and NADPH *(Cho et al. (1992))*. 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 is a constitutive, Ca^{2+}/calmodulin dependent enzyme 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 myocardium, skeletal muscle, or kidney *(Xu et al. (1996); Bredt et al. (1991))*.

Nitrite and NO

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

The NO^{2-} anions are unstable intermediates of oxidative transformation of NO to stable metabolite nitrate. It's concentration in the vascular system is associated with eNOS activity *(Gladwin et al. (2000))*. 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 *(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. The situation changes with the decrease of oxygen tension, suggesting nitrite physiological importance for the vasodilatation under these conditions *(Demoncheaux et al. (2002))*. 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 unless for extreme acidic conditions present in stomach or ischemic tissue *(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 xanthine oxidoreductase (XOR) *(Millar et al. (1998))*, deoxyhemoglobin *(Doyle et al. (1981))* 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 *(Kozlov et al. (1999))*.

Importantly, it was shown, that the eNOS, but not nNOS and iNOS, is 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

It was showed that several bacteria are capable of L-Arg conversion to citrulin with contemporaneous NO production *(Ninnemann et al. (1996))*. 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, is inhibited by common mammalian NOS inhibitors with close analogy to nNOS *(Chen et al. (1995))*. The genome sequencing of various bacteria showed the presence of similar genes in number of

other prokaryotes *(Zemojtel et al. (2003))*. These suspected proteins were annotated as close homologs of oxygenase domain of mammalian NOSs. Some of these proteins were subsequently purified and characterized *(Adak et al. (2002); Bird et al. (2002))*. 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))*. Some of the bacterial NOSoxy-like proteins have been shown to catalyze the oxidation of L-Arg or NOHA to citrulline and NO^{2-} *in vitro (Midha et al. (2005))*.

NO is generally considered to be toxic for the bacteria *(Kaplan et al. (1996))*. Although there are several reports describing involvement of bacterial NO-like proteins in the pathogenicity *(Kers et al. (2004))* or endurance in the extreme conditions *(Buddha et al. (2004))* of their bearers, the exact real role of prokaryotic NOS-like proteins and NO itself remains controversial.

Bacterial NOS analogs turned to be very useful for the study of their mammalian counterparts as well. The structural studies revealed that even though slight differences in the amino acid composition, the conformation of the active side 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))*.

Mitochondrial NOS

NO has profound effect on many physiological and pathophysiological processes in mitochondria, including reactive oxygen species (ROS) formation, energy metabolism and the regulation of oxygen consumption *(Giulivi et al. (2006))*.

The existence of mitochondrial nitric oxide synthase remains to be a controversial topic. Since the first reports suggested the presence of NOS in mitochondria based on anti-NOS antibodies staining mitochondria *(Bates et al. (1995))* 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 publications from different groups identifying mtNOS as eNOS *(Bates et al. (1996))*, iNOS *(French et al. (2001))* or nNOS *(Kanai et al. (2004))*. On the other hand, there is a number of works refuting the existence of mitochondrial NOS derived from the known three isoforms, and showing evidence of absence of NOS in mitochondria *(Brookes, P.S. (2004); Lacza et al. (2009))*. Additionally, NO can be produced inside mitochondria by other NOS independent pathways *(Basu et al. (2008))*.

Protein NOA1

In 2003 a new protein AtNOS1 from *Arabidopsis thaliana* was described as a potential nitric oxide synthase *(Guo et al. (2003)).* AtNOS1 shows no sequence similarity to known NOSs. The closest homolog to AtNOS is bacterial protein YqeH from *Bacillus subtilis*, belonging to a subfamily of cGTPases *(Morimoto et al. (2002)).* After reports showing its mitochondrial localization *(Guo et al. (2005); Zemojtel et al. (2006))* AtNOS1 was proposed to be a novel putative mitochondrial NOS, not related to the already known nitric oxide synthases.

It was showed recently, that AtNOA1 is not capable of arginine-dependent NOS activity but it is a functional GTPase instead *(Moreau et al. (2008))* and the crystal structure of the plant protein was solved *(Sudhamsu et al. (2008)).* The protein was renamed to NO-associated protein 1 (AtNOA1)

In the latest works NOA1 protein has been described to be essential for proper mitochondrial and chloroplast ribosome assembly and/or translation *(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 understanding 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 a special focus on hypoxic and anoxic conditions.

- to study the kinetic and dynamic properties of heme environment of nitric oxide synthase, using bacterial NOS-like proteins, that shows 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

Paper A. - 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 exhibit high homology with the oxygenase domain of mammalian NOSs, thus providing 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))*.

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.

The data from rebinding kinetics measurements 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 in the proximity of the active center. It was shown 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.

Paper B. - 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 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.

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

We showed that SA-NOS significantly differs from BA-NOS at the level of several important properties. In the native state, SA-NOS shows absorbance maxima characteristic for the hexacoordinated low-spin ferric state, whereas native BA-NOS absorbance maxima indicates pentacoordinated high-spin form identical to those of iNOS oxygenase domain containing BH_4 an L-Arg. *(Ghosh et al. (1997))*.

The affinity of substrate L-Arg for SA-NOS increases after the addition of BH_4, 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.

SA-NOS exhibits properties very similar to those of the oxygenase domain of inducible NOS (iNOSoxy) not containing H4B, as expected. By contrast, the properties of BA-NOS which look like those of BH_4-containing iNOSoxy are unexpected for a NOS-like protein not containing BH_4. The 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.

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.

Paper C - Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia

In this communication we scrutinized eNOS for a possible nitrite reductase capability by absorption spectroscopy, electron paramagnetic resonance (EPR) spectroscopy and electrochemical measurements.

We showed that purified eNOS reduces nitrite ions to NO under anoxic conditions *in vitro*. The nitrite reduction was shown to be performed by the

oxygenase domain of the protein, independently on the occurrence of pterin cofactor - BH_4.

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.

Paper D - 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, and also investigated the role of the heme domains in each of the isoenzymes in the nitrite reduction and compared the effect of anoxia at physiological (15 uM) and supraphysiological (500 uM) nitrite concentrations.

NO production from nitrite at physiological level under anoxic conditions was specific only to the endothelial NOS, whether nNOS and iNOS showed no nitrite reducing activity. High nitrite concentrations largely decreased the probability of NO release from the reduced heme of eNOS, as shown by ultrafast optical spectroscopy. The isolated 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). The chemiluminiscence measurement showed that hypoxic NO formation was enhanced approx. 3.6-fold compared to normoxia, and under completely anoxic conditions 8.3-fold higher actually. The NO formation was inhibited by addition of NOS inhibitor (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.

Paper E - Mammalian mitochondrial nitric oxide synthase: Characterization of a novel candidate

This work focused on the functional characterization of mammalian ortholog (murine AtNOS1) of the plant AtNOS1.

AtNOS1 was shown 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, and 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.

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.

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.

Paper G - Plant nitric oxide synthase: a never-ending story?

This short communication 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 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))*. 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.

Paper H - 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))* 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 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.

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 have 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 protein 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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List of the original communications

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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.
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