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# Endothelial Nitric Oxide Synthase

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## Abbreviations

NO – nitric oxide; NOS – nitric Oxide Synthase; NADPH – nicotinamide dinucleotide phosphate; FAD – flavin adenine dinucleotide; FMN – flavin mononucleotide; CaM - calmodulin; $BH_4$ – tetrahydrobiopterin; NHA – N-hydroxy-L-arginine; Arg – Arginine, L-NAME - $N^g$-nitro-L-arginine methyl ester
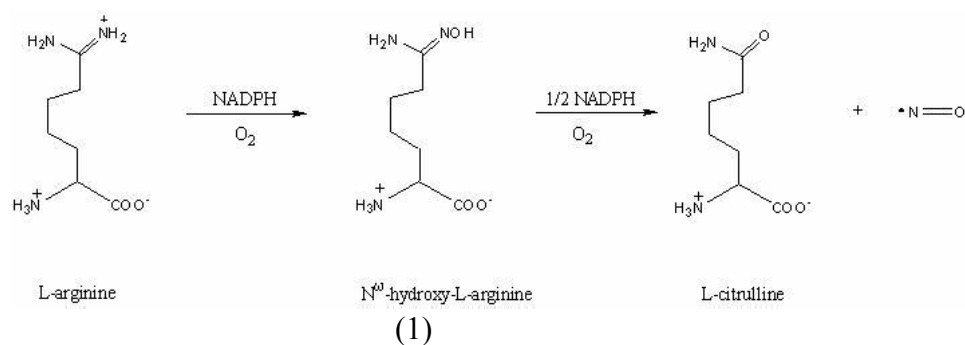
# Contents

# Abstract

The nitric oxide synthases (NOS) are hemoproteins with a cytochrome P450-like active site that catalyze the oxidation of arginine to nitric oxide and citrulline. Different isoforms of this enzyme has been discovered: eNOS, iNOS, nNOS and mtNOS. However, only first three were shown to be genetically different. This paper contains a brief comparison of these isoform and discussion of their catalytical mechanism. Second part of the paper specifically focuses on NOS III. Structural features, different levels of regulation and biological significance of this enzyme is discussed.

# Introduction

Nitric Oxide Synthases are the main mammalian enzymes that produce nitric oxide. These enzymes were shown to catalyze the following reaction [1].



$$\text{L-arginine} \xrightarrow[\text{O}_2]{\text{NADPH}} \text{N}^\omega\text{-hydroxy-L-arginine} \xrightarrow[\text{O}_2]{1/2\ \text{NADPH}} \text{L-citrulline} + \cdot\text{N}=\text{O}$$

(1)

Traditionally, three isoforms of NOS were identified in mammalian systems: NOS-I, NOS-II and NOS-III. They are encoded by different genes and have different tissue distribution and regulation. Recently fourth, mitochondrial isoform has been identified [36]. However this isoform was shown to be a subtype

of nNOS [36]. This paper will focus only on three "classic" isoforms. The main features of these
isoforms are summarized in Table 1.

**Table 1.**

| Isoform | NOS I (nNOS) | NOS II (iNOS) | NOS III (eNOS) |
|---|---|---|---|
| Cellular localization | Cytosolic | Cytosolic | Membrane bound |
| Tissue distribution | Nervous system, skeletal muscles | Ubiquitous | Vascular endothelium, airway epithelium |
| Regulation | $Ca^{2+}$/CaM | Cytokine inducible; $Ca^{2+}$ independent | $Ca^{2+}$/CaM |
| Expression | Constitutive | Inducible | Constitutive |
| Gene structure | 29 exons, 28 introns | 26 exons, 25 introns | 26 exons, 25 introns |
| Chromosomal location | 12q24.2-12q24.3 of chromosome 12 | 17cen-q11.2 of chromosome 17 | 7q35-7q36 of chromosome 7 |
| References | [17], [18] | [19], [20] | [22], [23] |

# Structure of NOS

NOS is a very complex enzyme that contains several cofactors and heme in the active site. Active NOS
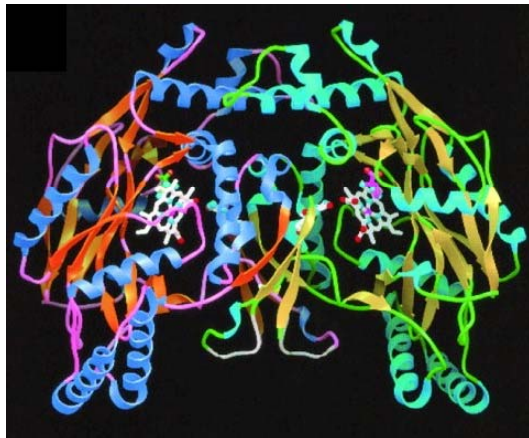has a dimeric form (Fig 1).



Fig 1. NOS dimer [Crane BR 1998]
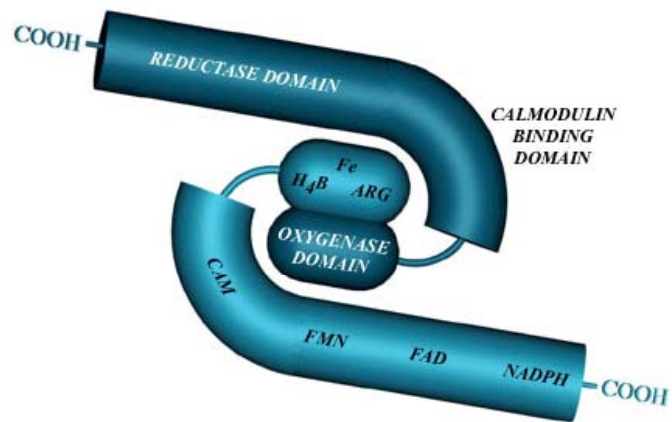


Fig 2. Domain structure of NOS dimer [online sources]

Each monomer has three domains: reductase domain, calmodulin binding domain and oxygenase
domain (Fig 2, Fig 3). Reductase domain consists of two subunits, one that binds NADPH and FAD and
the other that binds to FMN [24]. NADPH acts as a two electron donor. FAD and FMN serve as an
electron storage pool and transfer agent. $Ca^{2+}$/Calmodulin binding domain is very small (approximately

30 aminoacids). Oxygenase domain contains heme group, substrate binding site and binding site for tetrahydrobiopterin. It was shown that dimer interface contains a zinc tetrathiolate center, located at the bottom of the dimer interface. The zinc ion is tetrahedrally coordinated to two cysteins from each subunit. Structure of some cofactors for NOS is shown on figure 3.
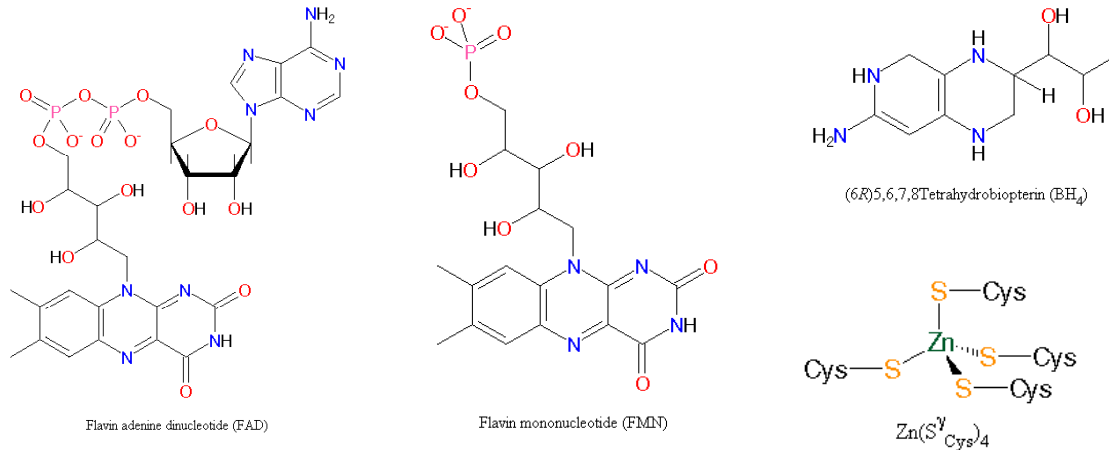


Fig. 3 Structure of cofactors for NOS [online sources]

# Catalytical mechanism of NOS

NOS catalyzes two sequential, mechanically distinct, heme based reactions. The first step is the hydroxylation of the amide nitrogen atom of arginine. The second step is conversion of NHA to citrulline and NO. NADPH serves as an electron donor for both reactions.
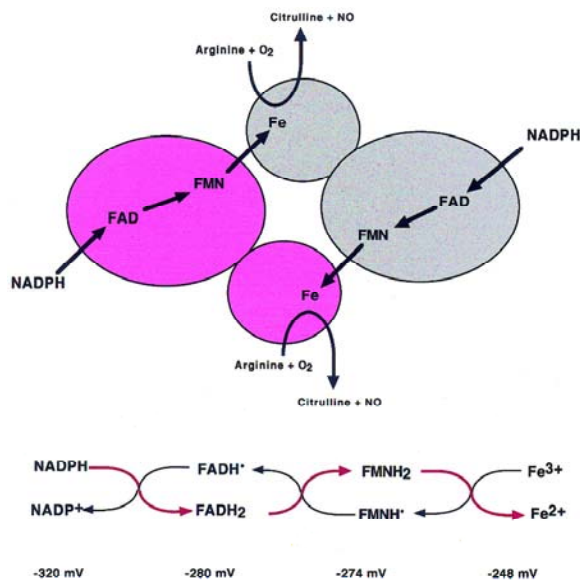


Fig. 4. Redox potential and direction of electron flow in nNOS

Upon binding of $Ca^{2+}$/calmodulin, FAD transfers reducing equivalents from NADPH to FMN, which then reduces heme in oxygenase domain of opposite monomer (Fig. 4). The ferrous form of the heme binds oxygen to form stable oxy complex (Fig 5). Dissociation of this species is a source of superoxide anion in uncoupled NOS. The feroxy complex is then protonated and the oxygen – oxygen bond breaks to form a very active intermediate, that performs the two electron oxidation of arginine to NHA. Second oxidation reaction involves formation of ferric-peroxy intermediate that catalyses this reaction. Ferric iron is reduced to ferrous and oxygen binds.

This form of the enzyme then reacts directly with NHA to form an NHA radical and haem-peroxy complex. The NHA radical and the peroxy complex then react with each other in a "radical rebound" mechanism to generate citrulline and NO and regenerate the ferric haem iron [24]
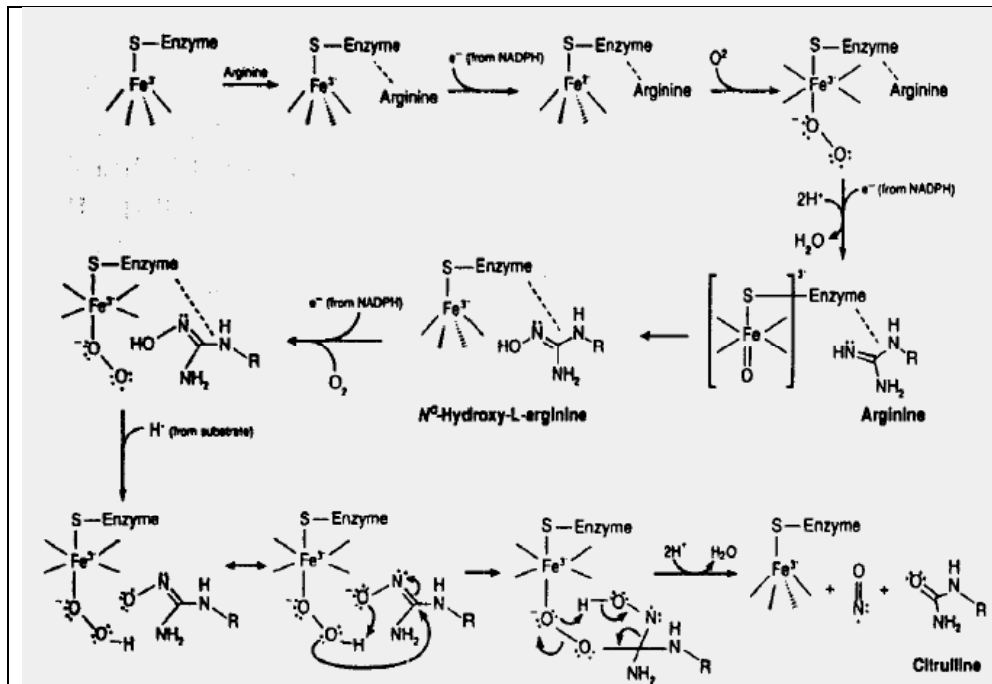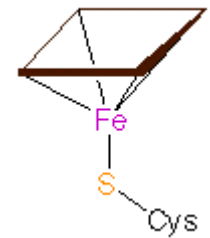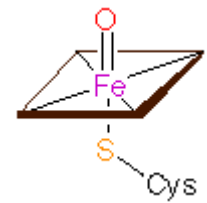


Fig 5. Catalytical mechanism of NOS [Boga et al 1998]

Pentacoordinate

Hexacoordinate

Fig 6. Haem iron coordination

# Endothelial NOS

Endothelial NOS is the main source of NO in vasculature. Deregulation of eNOS is involved in pathogenesis of many cardiovascular diseases: atherosclerosis, hypertension, diabetes, hypercholesterolemia, hyperhomocysteinemia etc [24].

The eNOS knockout mouse (eNOS-/-) have the following phenotype: hypertension, poor vasorelaxing activity, increased neo-intimal proliferation and an increased contractile response to $\beta$-adrenergic agonists. Furthermore, it has been described that these animals have developmental growth problems, abnormal aortic valves, and limb abnormalities. These mice also exhibit impaired wound-healing capacity and a poor response to growth factor-stimulated angiogenesis. ENOS -/-mice also have impaired endothelium-dependent vasodilation in the pulmonary circulation [24].

Three different polymorphic variations in the human eNOS gene have been identified. Two single nucleotide substitutions have been identified within the introns, one in intron 18, Ala27Cys, and one in intron 23, Gly10Thr. Some variable repeats have also been located in introns 4, 13, and 23. Although a

normal functional enzyme is formed, the polymorphisms could lead to altered transcription and/or processing rates, thereby interfering with normal enzyme function. Individuals with a polymorphism in intron 13 have a higher risk of coronary artery disease [24]

The endothelial nitric oxide synthase (eNOS) is a dimer consisting of two identical monomers of 134 kD. The gene encoding for the eNOS monomer is located on chromosome 7q35-36 and contains 26 exons, spanning 21 kb. Domain structure of eNOS is shown on the Fig 5.
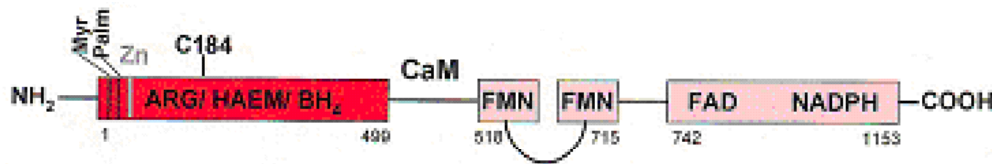
Fig 7. Domain Structure of eNOS [25].

The main difference between this isoform of NOS is palmitoylation and myristoylation sites. Palmitoylation and myristoylation are required for targeting of eNOS to plasma membrane. Endothelial NOS has different levels of regulation that are summarized in the Fig 8.
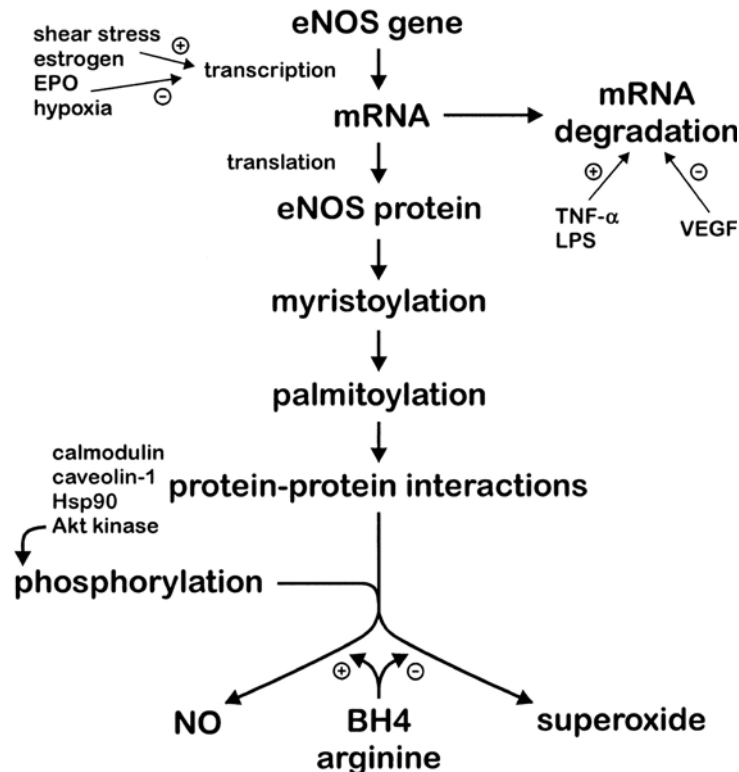
Fig. 8 Different levels of eNOS regulation [17]

The eNOS enzyme is only fully functional in a dimeric form. Dimerization of eNOS starts with the binding of haem without haem, the eNOS enzyme exists only as a monomer. The binding of haem and the formation of a dimer make it possible for tetrahydrobiopterin to bind to the eNOS dimer, which leads

to the formation of a stable dimer. Stabilization of the dimer is also dependent on zinc ions, which maintain the integrity of the $BH_4$ binding site. The functional activity of the eNOS dimer is dependent on the number of $BH_4$ molecules bound. An eNOS dimer without $BH_4$ is capable of producing $O_2^-$; the binding of one $BH_4$ molecule results in an eNOS dimer capable of producing both NO and $O_2^-$. High levels of $BH_4$ result in a saturated dimer, which acts purely as an NO synthase [24].

It is thought that most eNOS is located in the caveolae, where it is bound to caveolin, a resident coat protein. The caveolin binding leads to inhibition of eNOS activity by interfering with the CaM binding and electron transfer to the haem subunit. A rising intracellular level of free $Ca^{2+}$ causes the formation of $Ca^{2+}$/CaM complexes, resulting in the binding of the enzyme and consequently the dissociation of caveolin. The eNOS enzyme is now activated and remains so until intracellular $Ca^{2+}$ levels drop and the $Ca^{2+}$/CaM complex is subsequently replaced by caveolin. The activated eNOS enzyme is productive as long as the substrates L-arginine, oxygen, and NAPDH are present. Altered lipid composition in the caveolae can displace the eNOS enzyme, thereby altering the eNOS activation process.

No specific inhibitors for eNOS are available so far. The most often used inhibitor for all isoforms of NOS is L-NAME.


# Conclusions

It is difficult to underestimate significance of endothelial NOS. This enzyme is the major source of NO in vasculature. It is one of the most complex enzymes ever known. Activity of eNOS could be regulated on different levels. Impairment of eNOS function is involved in pathogenesis of many diseases. Studying eNOS could have a lot of therapeutic implications. Endothelial NOS gene therapy could be used a potent therapeutic tool in the future.

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