

NITRIC OXIDE: ONE OF THE MORE CONSERVED AND WIDESPREAD SIGNALING MOLECULES

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1. ABSTRACT

After the discovery of the vasodilatory functions of nitric oxide (NO), many signaling mechanisms involving NO were identified through experiments on mammals. NO activates soluble guanylyl cyclase to induce the formation of cGMP, stimulates ADP-ribosylation of GAPDH to alter cell energy production, and combines with superoxide to generate peroxynitrite. It then became clear that NO was a major messenger molecule in mammals, involved in the regulation of blood vessel dilatation, immune function and neurotransmission in the brain and peripheral nervous system. The wide spectrum of physiological effects of NO in mammals prompted researchers to look for the presence of NO in vertebrates and invertebrates. Parallel findings on the presence of NO signaling in vertebrates and invertebrates were observed, and then NO was found to be a signaling molecule widely spread throughout the metazoan kingdom and whose functions were highly conserved during evolution. These features were extended to the entire animal kingdom after the discovery of NOS activity in protozoa, yeasts and bacteria. Recently, the involvement of NO and NOS in plant disease resistance to infection was documented and many close similarities were detected between NO-dependent signaling mechanisms involved in plants and those identified in animals. All of these results indicated that NO is one of the earliest and most widespread signaling molecules in living organisms. This short review was aimed at marshalling recent information that led to this conclusion.

2. INTRODUCTION

Nitric oxide (NO) was a component of the Earth's primitive atmosphere, but for metazoan biologists it is a young molecule that appeared only 14 years ago. Indeed, although NO was known to be a central player in bacterial bioenergetics (1), a biological function of NO in eukaryotes was observed first by Palmer *et al.* (2) in 1987. These authors showed that NO and an endothelium-derived relaxing factor (EDRF) discovered

7 years ago (3) have identical pharmacological properties and that endothelial cells in culture released NO. Then very intense research into the biological functions of NO in living organisms began and the number of publications dealing with physiological and pathophysiological roles of NO have increased exponentially year by year.

3. MAMMALS

NO is a water- and lipid-soluble radical gas that can be involved in redox reactions, giving rise to nitrosylated derivatives, and it combines with other radicals or transition metal ions (Figure 1). Moreover, NO is electrically neutral and readily diffuses through membranes (4).

NO is produced during the conversion of L-arginine to L-citrulline (5) by a family of enzymes, i.e. NO-synthases (NOS) (Figure 2). In mammalian cells, three distinct NOS isoforms (Table 1) have been isolated and represent the products of three different genes (Figure 3). Type I (neuronal) and type III (endothelial) isoforms termed constitutive NOS, are constantly expressed and Ca²⁺-dependent. The type II (macrophagic) isoform is not typically expressed in resting cells, must be induced by cytokines and microbial products such as lipopolysaccharides, and is Ca²⁺-independent. All NOS forms are homodimers of subunits which range between 130 and 160 kDa and have a bidomain structure (Figure 4): an oxygenase domain within the amino-terminal half and a reductase domain within the carboxy-terminal half. They use three substrates: L-arginine, NADPH and O₂ and require five cofactors: FAD, FMN, calmodulin (CaM), tetrahydrobiopterin (BH₄) and heme (6, 7).

Non-enzymatic NO production was recently detected in humans (8, 9), it results from the chemical reduction of inorganic nitrites and is effective in stomach, skin surface and ischemic heart.

Three major molecular targets of NO have been detected: soluble guanylyl cyclase (CGs), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and superoxide. They

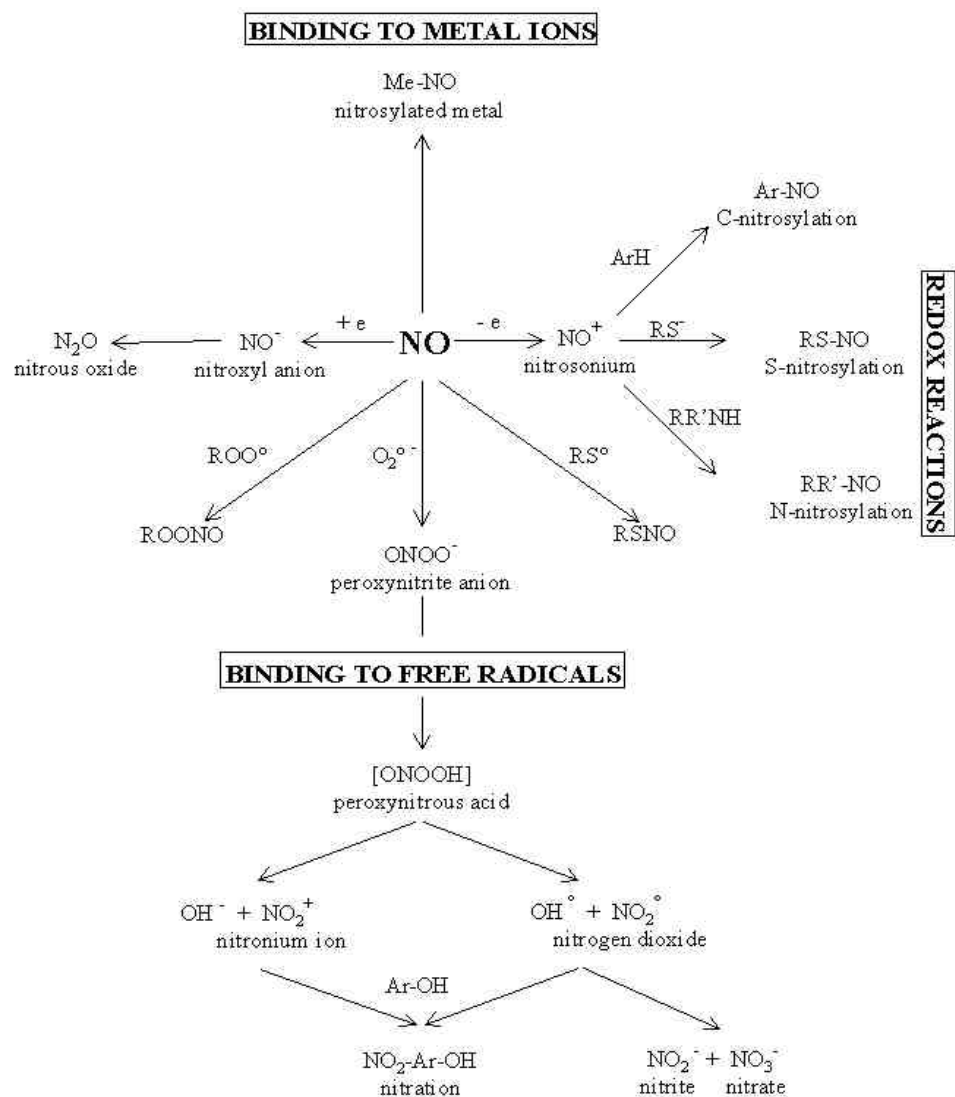


Figure 1. Chemical reactivity of nitric oxide

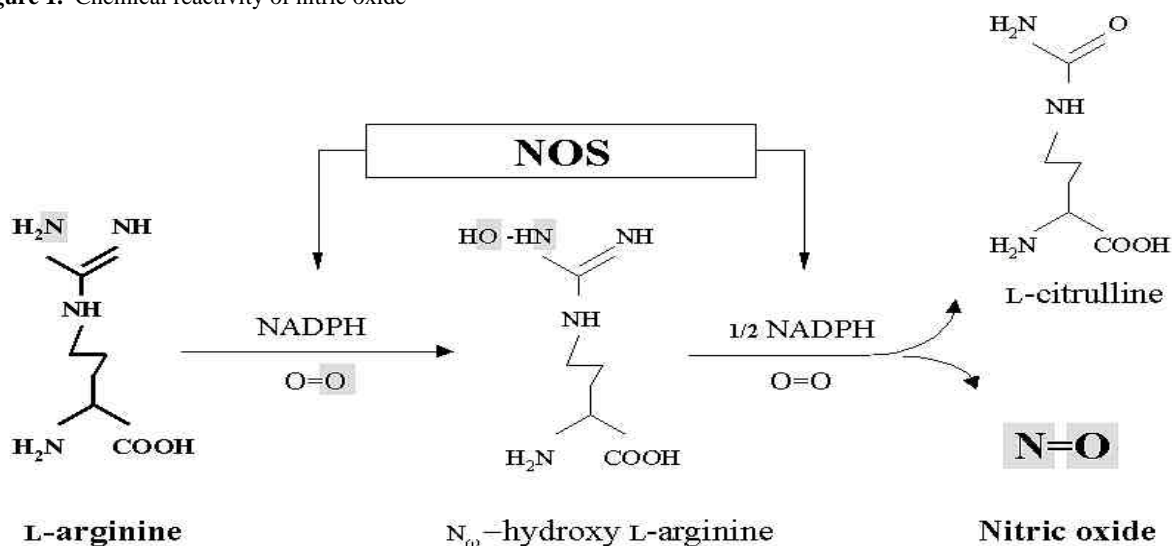


Figure 2. Biosynthesis of nitric oxide. The nitric oxide radical is generated through five-electron oxidation of a guanidino nitrogen of L-arginine via the N^G-hydroxyl-L-arginine intermediate.

Table 1. Characteristics of the three human NOS isoforms

Human NOS type	I (neuronal)	III (endothelial)	II (macrophagic)
V _{max} (μmol mg ⁻¹ min ⁻¹)	0.3 - 3.4	0.015	0.9 - 1.6
mRNA size (kb)	10.0	4.7	4.4
Protein size (kDa)	160	133	130
L-Arg Km (μM)	2 - 4.3	2.9	2.3 - 2.8
MeArg IC ₅₀ (μM)	1.6	0.9	7.4
Ca ²⁺ EC ₅₀ (μM)	0.2 - 0.6	0.3	No dependence
Regulation of expression	constitutive	constitutive	inducible
Aminoacid identity (%)	51		57
	54		

correspond to the three fields of NO reactivity: binding to metal ions, nitrosylation and combination with another radical.

CGs is a heme protein which catalyzes the formation of the guanosine cyclic 3'-5'-monophosphate (cGMP). NO binding to the heme iron of GCs leads to activation of the enzyme by formation of a nitrosyl-heme complex which dislocates the iron-heme complex. This induces a conformational change in GCs which stimulates its catalytic activity. GCs generates cGMP, which in turn specifically regulates protein phosphorylation, ion channel conductivity and phosphodiesterase activity. NO is the most potent and effective activator of GCs. This NO-cGMP signal transduction system accounts for the observed role of NO in central and peripheral nervous systems as well as in cardiovascular, renal and pulmonary systems (10).

- GAPDH (in brain, red blood cells and platelets) is a glycolytic enzyme which normally oxidizes glyceraldehyde phosphate and transfers electrons to NAD⁺. NO alters this reaction sequence by ADP-ribosylating the enzyme (11, 12). ADP-ribose resulting from the cleavage of NAD⁺ is transferred to a GAPDH S-nitrosylated cysteine and causes permanent inhibition of the enzyme.

- Superoxide produced during the burst of phagocytes is another major molecular target of NO. The combination of both radicals generates peroxynitrite (Fig.1), a potent oxidizing and nitrating agent capable of attacking and modifying proteins, lipids and DNA and depleting antioxidant defenses (13, 14).

Experiments by Kamosinska *et al.* (15) on lung epithelial cells give an example of interaction between NO metabolic pathways. They showed that activation of the whole-cell current *via* NO/cGMP is inhibited by the peroxynitrite generated subsequently to the cytokine-induced expression of type II NOS.

All studies have demonstrated that NO plays a crucial role in physiological and pathophysiological processes in mammalian bodies: neurotransmission, neuromodulation, vasodilatation, vasoprotection, secretory

control, intestinal relaxation and antipathogen defense (16-25).

But how can NO also function safely as a messenger and neurotransmitter when produced at low concentrations and becomes cytotoxic, *via* peroxynitrite formation, when produced at high concentrations?

This paradoxical situation was clarified by kinetic studies (26). Indeed, the reaction rate between NO and superoxide to form peroxynitrite is 3-fold faster than the rate of superoxide dismutation by superoxide dismutase (SOD, the enzyme that protects tissues against superoxide). When SOD concentrations remain constant, as is generally the case in healthy cells and tissues, the NO concentration is thus the main driving force for peroxynitrite formation. NO produced as a continuous flux by unregulated Ca²⁺-independent type II NOS is then oriented toward defensive activity.

Moreover, the three NOS isoforms present in mammals were found in other vertebrates.

4. OTHER VERTEBRATES

The L-arginine-NO pathway observed in mammals has also been demonstrated in fishes (27, 28), for example, neuronal type I NOS activity was detected in the brain and spinal cord of an amphibian, the frog *Rana perezi* (29, 30), as well as in all regions of the gut of a reptile, the estuarine crocodile (35). This showed that the gastrointestinal motility in reptiles is under NO control. Inducible type II NOS was characterized in fish macrophages. The NOS from carp phagocytes (36-38) has 57% sequence identity to human enzyme and contains binding sites for the same cofactors as mammalian NOS. In rainbow trout (36-38), a 130 kDa Ca²⁺-independent NOS was found in liver and head kidney. In chicken, an increase in nitrite production by splenic macrophages was observed during the course of coccidiosis (39, 40). Endothelial type III NOS was found in retina Muller cells of all phylogenetic classes of vertebrates (41, 42). In day-old chicks, Rickard *et al.* (43) observed significant memory loss for 40 min post-training following the intracranial administration of diphenylene iodonium (DPI) which is known to specifically inhibit endothelial type III and inducible type II NOS. On the contrary, no

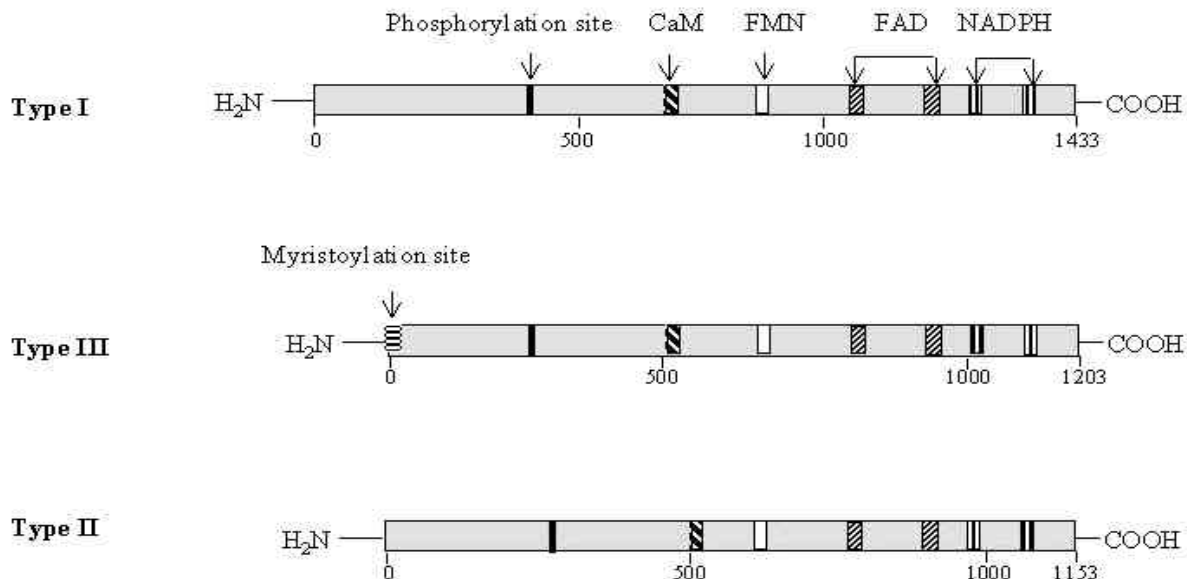


Figure 3. The primary structure of the three human NOS isoforms (modified from Knowles and Moncada, 1994 (118)). amphibians (29-31), reptiles (32) and birds (33, 34).

significant effect on memory formation was observed after injection of three neuronal type I NOS specific inhibitors (3-bromo-7-nitroindazole, N-propyl-L-arginine and S-methyl-L-thiocitrulline), at least until 2 h post-training. The authors concluded that the role played by endothelial type III NOS in the memory formation is distinct from any role that may be played by neuronal type I NOS.

Therefore, studies of NOS activity in lower vertebrates confirmed the results obtained with mammalian cells and tissues and gave insight into the physiological role of NO in two new domains: memory formation and light adaptation of retina. This latter NO function occurs *via* the NO-sGC-cGMP cascade as shown by Blute *et al.* (42, 44) in turtle retina.

5. INVERTEBRATES

Soon after the detection of NO with EDRF (3), Radomski *et al.* (45) demonstrated the role of NO and cGMP in the regulation of platelet adhesion in mammals. Widening their studies to hemocytes from an arthropod, the horseshoe crab *Limulus polyphemus* (46), these authors observed that aggregation of these hemocytes is under control of the NO they produce. This result provides the first clear evidence of NO production in invertebrates. Since the horseshoe crab is a living fossil directly related to trilobites that lived 500 million years ago, it appears that the NO-cGMP signaling cascade has been strongly conserved through the evolution process.

Since then, a number of studies were carried out on invertebrates. After the histochemical detection of NADPH-diaphorase activity by Elofsson *et al.* (47) in tissues of annelids, mollusks, arthropods, echinoderms and urochordata, several studies provided evidence of

the extensive presence of NOS in invertebrate tissues (48-51).

As for vertebrates, histochemical, physiological and biochemical data show that the L-arginine-NO pathway is involved in many physiological functions in invertebrates. Some well documented reviews (52-56) summarize the knowledge about the functional roles of NO in invertebrates. The most specific contributions of invertebrate studies to knowledge on physiological role of NO deal with development, synaptic plasticity, learning and sensorial functions associated with feeding and olfaction. This is probably due to the accessibility and simplicity of invertebrate nervous systems, which provide accurate information on all neurons and their functions. Some recent reports suggest that NO could play similar roles in vertebrates (57-60).

Moreover, recent studies on *Drosophila* development (61) have firmly established the role of NO in the control of the balance between cell proliferation and differentiation previously suggested by works on mammalian embryonic development (62).

Interestingly, the studies of Colasenti *et al.* (63, 64) revealed the presence of the NO-cGMP pathway in the freshwater coelenterate *Hydra*. Since *Hydra* is the most primitive organism with a nervous system, these observations support high evolutionary conservation of the NO-cGMP pathway.

Some NOS from invertebrates have been characterized (50, 55, 64, 65). They exhibited properties similar to that described for vertebrate enzymes. i) They used L-arginine as substrate, ii) formed NO stoichiometrically with L-citrulline, iii) were competitively inhibited by L-arginine analogs, and iii) had apparent

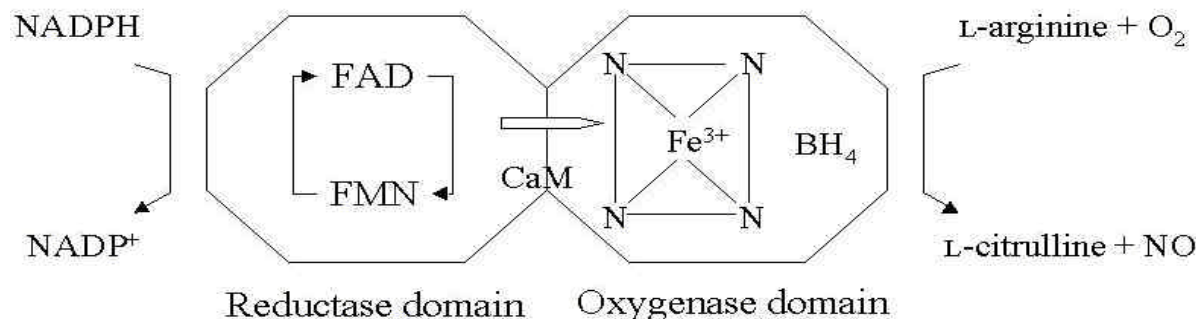


Figure 4. Schematic representation of the nitric oxide synthase monomer. Nitric oxide synthases are dimers whose monomers are all composed of two domains; they use three substrates: L-arginine, NADPH and O₂ and require five cofactors: FAD, FMN, calmodulin (CaM), tetrahydrobiopterin (BH₄) and heme.

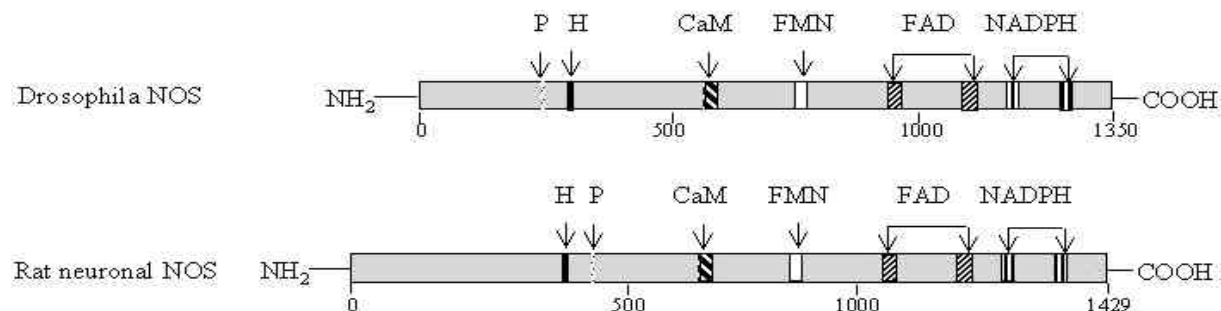


Figure 5. Comparison of the primary structure of NOS from *Drosophila* and rat brain (modified from Regulski and Tully, 1995 (66)).

molecular weights in the 130-150 kDa range in SDS-polyacrylamide gel electrophoresis. However, it is not clear if all isoforms described in vertebrates are present in invertebrates.

The *Drosophila* NOS gene cloned in 1995 by Regulski and Trulli (66) encoded a protein having 43% amino acid sequence identity to rat type I NOS (Figure 5).

Some years later, Ogunshola *et al.* (67) characterized NOS from locust *Schistocerca* and pond snail *Lymnaea*. They have more than 50% amino acid sequence identity to vertebrate type I NOS and are calcium dependent (68).

Yuda *et al.* (69, 70) cloned and expressed a Ca²⁺-dependent NOS present in the salivary and CNS of *Rodnius prolixus*, a blood sucking arthropod vector of Chagas' disease. This enzyme was found to have a 130 kDa apparent molecular weight, and its amino acid sequence lacked part of the N terminal domain when compared to mammalian type I and II NOS.

In a comparative analysis on NOS activity performed in several mollusks, Moroz *et al.* (51, 71) reported the presence (in *Pleurobranchia* and *Aplysia*) of NOS activities inhibitable by trifluoroperazine, a calmoduline inhibitor. This suggests the presence of a new NOS isoform since trifluoroperazine is ineffective in blocking the activity of the three NOS isoforms described in mammals.

The involvement of NO and NOS in invertebrate defensive systems is not as well documented.

Ottaviani *et al.* (72) showed that the bacterial clumping activity of *Mytilus edulis* haemolymph stimulated by LPS was selectively and significantly reduced by NOS inhibitors. This indirectly demonstrates that hemocytes of *M. edulis* make and use NO as a bactericidal agent to kill phagocytosed organisms.

Torreilles and coworkers, by spectroscopic measurements (73-75) or using an anti-nitrotyrosine antibody (76), reported that upon challenge with zymosan *M. galloprovincialis* and *Crassostrea gigas* hemocytes generate peroxynitrite.

Franchini *et al.* (77) reported that immunocytes from *Viviparus ater* exhibit NOS activity when stimulated with *E. coli*. Calcium deprivation promoted 30% inhibition of NOS activity, suggesting that a Ca²⁺-independent NOS isoform is present in immunocytes next to the Ca²⁺-dependent isoform.

Recently, in two arthropods, *Drosophila melanogaster* and *D. teissiere*, Nappi *et al.* (78) observed enhancement of NO production at a time when eggs of *Leptopilina boulardi*, a *Drosophila* parasite, were destroyed by hemocyte-mediated melanotic encapsulation. This demonstrated that NO is involved in the *Drosophila* defense system against eukaryotic parasites.

Table 2. Characterization of NOS activity in some bacteria and yeast species

Protozoa	Molecular weight (kDa)	Mammalian antibody crossreactivity	Ca ²⁺ dependence	N ^G -nitro-arginine Ki (μM)	L-Arg Km (μM)
<i>Trypanosoma cruzi</i>	-	-	yes	40 (NMMA IC50)	-
<i>Leishmania donovani</i>	110	Type I	yes	6.8	4.9
<i>Plasmodium falciparum</i>	< 100	Type II and III	no	3.5	3.8
Yeasts					
<i>Saccharomyces cerevisiae</i>	60	Type I	yes	-	-
<i>Candida tropicalis</i>	-	-	-	-	-
Bacteria					
<i>Nocardia</i>	519	-	yes	14.6	8.2

The identification of NOS in mollusks, crustaceans, arthropods and other invertebrates confirms the wide distribution of the L-arginine-NO signaling system in metazoan phyla and its strong evolutionary conservation (79, 80).

6. LOW EUKARYOTIC ORGANISMS

Soluble NOS activities were reported to be present in lower eukaryotic organisms, i.e. protozoan parasites and yeast. In *Trypanosoma cruzi* epimastigote forms (81, 82), NOS was localized on the inner surface of cell membranes and in free cytosolic clusters in the body, the flagellum and apical extreme. NMDA and excitatory amino acids such as glutamate activated NOS and increased intracellular levels of cGMP as described for mammalian neural cells.

Soluble NOS activity requiring NADPH was purified 2800-fold from the protozoan parasite *Leishmania donovani* (83). Its Western blot analysis with NOS type I antibody suggested a strong similarity with the neuronal NOS isoform from mammals.

Moreover, a Ca²⁺-independent NOS activity was detected in *Plasmodium falciparum* infected red blood cells (84).

The three enzymes showed apparent molecular weights of < 100 kD (Table 2).

NOS activities were also detected in yeast *Saccharomyces cerevisiae* (85) and *Candida tropicalis* (86).

The presence of NOS activities in species phylogenetically distant from mammalian species confirms the very ancient origin of NO synthesis in living organisms and the strong evolutionary conservation of the NO transduction pathway.

7. PLANTS

As we have previously shown, NOS are the almost exclusive sources of NO in animals. In plants, on the contrary, three major origins of NO production are effective: i) the activity of nitrate and nitrate reductase, ii) the non-enzymatic light-mediated NO₂⁻ conversion by carotenoids, and iii) the activity of mammalian-type NOS (Figure 6).

The uptake of atmospheric NO (which results from N₂O oxidation into the atmosphere, and is one of the elements of the Earth's nitrogen cycle) by plant foliage and its deleterious consequences have been known for many years (87, 88). However, NO synthesis in plants has been demonstrated recently (89), and studies on the physiological role of NO in plants have just begun (90). However, mounting evidence suggests that, as in animals, NO is an ubiquitous signal in plants.

Noritake *et al.* (91) observed, in potato tuber tissue, the induction of phytoalexin accumulation by NO, while experiments performed by Gouvea *et al.* (92) and Riberio *et al.* (93) strongly suggest that NO controls root growth and development in maize.

Moreover, an effect of NO on peroxidases involved in the cell wall lignification in plants was reported by Ferrer and Ros Barcelo (94).

Evidence of the involvement of NO and NOS in plant disease resistance to infection was recently obtained in tobacco (95-99) and soybean (100).

In the light of Durner's results and the other published data, Durner *et al.* (101) proposed a model in which reactive oxygen species and salicylic acid act synergistically with NO to activate the plant defense response to pathogens and promote host cell death.

Indeed, the most powerful weapon in plants against pathogen attacks is rapid localized cell death at the site of infection (102). This cell death response, called the hypersensitive response, deprives pathogens of access to a nutrient source and limits their proliferation.

As emphasized by Cohn *et al.* (103), signaling pathways involved in plant disease resistance mechanisms are just beginning to be unraveled, but many strong similarities between these mechanisms and those involved in innate immunity in animals have already been noted (104).

8. BACTERIA

As claimed by Zumft (1), the central role played by NO in bacterial energetics and in the global N cycle, vital to all organisms, has been known for a long time (105). Two pathways for NO biosynthesis have been observed: nitrification, which corresponds to oxidation of NH₄⁺ to NO₃⁻,

Nitric oxide in living systems

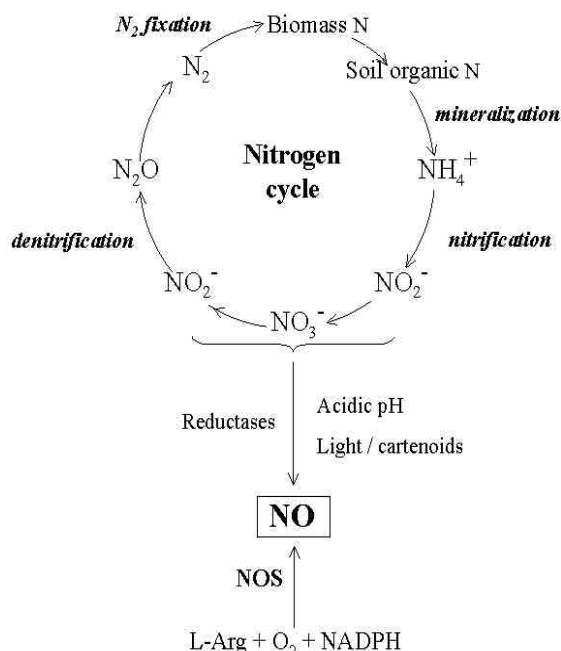


Figure 6. Enzymatic and non-enzymatic sources of NO in plants

and denitrification, which corresponds to the reduction of NO_3^- and NO_2^- to N_2 (106-113).

More recently, a third NO biosynthesis pathway was also detected in bacteria. Chen *et al.* (114, 115) isolated and purified, in *Nocardia* bacteria, NOS which required for the same cofactors as mammalian enzymes to ensure their activity, but they were found to have a 51.9 kDa apparent molecular weight.

9. PERSPECTIVES

NO is an odd endogenous control agent:

- for its nature. NO is basically a gas found to be present everywhere in animal bodies. Locally produced by many types of cells, it is an unstable radical with a biological half-life of only few seconds. NO acts as a transmitter, by diffusing in the immediate vicinity of the cells from which it has been released, and as a hormone, by binding to transporters and entering the blood stream to remote from the release site. Moreover, when NO acts as an intercellular messenger, it does not react as classical messengers, by noncovalent binding to specific receptors through complementarity of shape, but rather by covalent binding to its targets on the basis of their redox potential.

- for its large number of physiological roles. NO serves as a multifunctional messenger affecting many physiological processes such as apoptosis (116), regulation of vascular tone, macrophage-mediated resistance to infection (117), control of synaptic connectivity during development and synaptic plasticity in adults, mediation of olfactory memory

formation, regulation of the balance between cell proliferation and differentiation, and thus control of the shape and size of adult animal organs. NO has a key role in many physiological processes and could thus be considered as the most ubiquitous biological messenger in living organisms.

- for its occurrence and common functions in virtually all living systems. NOS isoforms, with 150-160 kDa molecular weight and large conserved amino-acid sequences, have been identified in mammals, vertebrates and invertebrates. NOS were also characterized in protozoa and prokaryotic cells but with molecular weights lower than 100 kDa and amino acid sequences differing from those of multicellular animal organisms. The main functional roles and transduction pathways of NO described for mammals have been found in other vertebrates as well as in invertebrates. Few data are available for protozoa, bacteria and plants, but it is probable that future research will reveal similar signaling functions in these organisms. The diffusion of NO as a signaling molecule in living organisms from mammals down to bacteria and plants suggests that NO could be one of the most widespread biological messengers in all the living species.

- for the high conservation of its main signaling pathways during evolution. Comparative investigations on the signaling functions of NO in different organisms have also provided insight into the early evolutionary roles of NO. Indeed, evidence on the involvement of NO in the cellular defense system of *Limulus polyphemus* (an arthropod which has not evolved in 500 million years), as well as in the feeding response of *Hydra* (the most primitive organism possessing a nervous system), suggest that NO could be one of the earliest biological signaling molecules.

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