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Nitric Oxide in Invertebrates

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Abstract

Nitric oxide (NO) is considered an important signaling molecule implied in different physiological processes, including nervous transmission, vascular regulation, immune defense, and in the pathogenesis of several diseases. The presence of NO is well demonstrated in all vertebrates. The recent data on the presence and roles of NO in the main invertebrate groups are reviewed here, showing the widespread diffusion of this signaling molecule throughout the animal kingdom, from higher invertebrates down to coelenterates and even to prokaryotic cells. In invertebrates, the main functional roles described for mammals have been demonstrated, whereas experimental evidence suggests the presence of new NOS isoforms different from those known for higher organisms. Noteworthy is the early appearance of NO throughout evolution and striking is the role played by the nitrergic pathway in the sensorial functions, from coelenterates up to mammals, mainly in olfactory-like systems. All literature data here reported suggest that future research on the biological roles of early signaling molecules in lower living forms could be important for the understanding of the nervous-system evolution.

Index Entries: Nitric oxide; NO synthase; invertebrate; evolution; nervous system.

The Arginine/NO Pathway: General Features and Physiological Roles

Nitric oxide (NO), a gas previously considered to be an atmospheric pollutant, is a major messenger molecule playing key roles in many physiological and pathological processes. NO is an unstable nitrogen radical generated in mammalian cells by the concomitant conversion of arginine into citrulline through the enzyme NO synthase (NOS). There are at least three distinct isoforms of NOS present in mammalian cells. Two enzymes, the neuronal and the endothelial Ca^{2+}-dependent isoforms (NOS-I and NOS-III, respectively) are constantly expressed and termed constitutive NOS (cNOS). The third enzyme is an inducible Ca^{2+}-independent isoform (iNOS or NOS-II), which is expressed after stimulation with *Escherichia coli* lipopolysaccharide (LPS) and/or cytokines, such as interferon-γ (IFNγ), interleukin-1β (IL-1β), or tumor necrosis factor-α (TNF-α). The induction of human NOS-II occurs at the transcriptional level (de Vera et al., 1996) and is mediated by the early activation of some nuclear factors such as NF-κB (Goldring et

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al., 1995), and interferon regulatory factor-1 (Kamijo et al., 1994).

However, NO is a double-edged sword. In fact, NO generated at low levels by cNOS plays important roles in physiological processes as neurotransmission or vascular regulation. In neurons, the NOS-I is activated by the glutamatergic pathway stimulation, as after and consequent to a raised cytosolic Ca^{2+} influx (Garthwaite, 1991; Snyder and Bredt, 1991). NO can exert its biological activity via the stimulation of the soluble guanylate cyclase (Garbers, 1992), thus leading to an increase in cyclic GMP (cGMP). On the other hand, uncontrolled and massive NOS-II-induced NO production is implicated in host defense, immunological reactions, and nonspecific immunity. Furthermore, it is involved in the pathogenesis of conditions including septic shock, stroke, diabetes, inflammation, AIDS, and neurodegeneration.

NO in Invertebrates: First Observations

NOSs were originally described in mammalian tissues. Since 1992, increasing evidence has demonstrated the presence of the L-arginine-NO pathway in all vertebrates such as fish (Li and Furness, 1993; Schober et al., 1993; Huque and Brand, 1994; Schoor and Plumb, 1994), cyclostomes (Schober et al., 1994; Zielinski et al., 1996), amphibians (Li et al., 1993), reptiles (Kinney and Slater, 1993), and birds (Lyon and Hinshaw, 1993). In these vertebrate groups, NO has been suggested to play roles both in immunological defense and neurotransmission.

However, many years ago, before NO was recognized as a signaling molecule, D'Alessio et al. (1982) demonstrated that L-arginine is required for memory consolidation in the praying mantis, thus suggesting a role of this NO-generating amino acid in an insect learning mechanism.

The first clear evidence of NO production in invertebrates was provided by Radomsky et al. (1991). The horseshoe crab *(Limulus polyphemus)* hemocyte was found to produce NO, which controls cell aggregation in the same way as in mammalian platelets. As suggested by evidence in fossils, this arthropod has not evolved in 500 million years, thereby supporting a strong evolutionary conservation of this signaling pathway.

Soon afterwards, a number of studies were carried out on invertebrates. Using the histochemical method for NADPH-diaphorase, which is considered to be colocalized or identical to neuronal NOS, Eloffson et al. (1993) investigated the presence of NOS activity in several invertebrates, from coelenterates to higher forms. These authors reported a positive reaction in nervous and nonnervous tissues of Annelids, Mollusks, Arthropods, Echinoderms, and Urochordata, but not in Coelenterates, Platyhelminthes, and Nematodes. In this respect, they concluded that, contrary to other neurotransmitters, NO should be confined to more evoluted invertebrates.

After these pioneering observations, several studies provided evidence of an extensive presence of NOSs in some tissues, particularly in mollusks and artropoda. For these groups, the presence and some possible roles of NO are now well understood. Taken together, literature data suggest that as for mammals, L-arginine-NO pathway is involved in neurotransmission, neuromodulation, smooth muscle activity, and immunological defense mechanisms. A very interesting role of NO was found by Ribeiro and colleagues (Ribeiro et al., 1990) in the bloodsucking insect *Rhodnius prolixus,* which induces vasodilatation in the host by injecting a NO-loaded heme protein. Recent observations have demonstrated the involvement of NO in functions such as cellular proliferation, differentiation, and predation.

It was only in more recent years that, using biochemical or physiological approaches, the presence and possible roles of NO were also analyzed in other invertebrate groups, such as annelids, echinoderms, nematodes, flatworms, coelenterates, and even in protozoa and bacteria (Tables 1 and 2).

Table 1
Presence of Nitric Oxide in Invertebrates

Animals	Determination	References
Mollusca		
Lymnaea	LFB	Moroz et al., 1993; 1994; 1995; Moghadam et al., 1995; Elphick et al., 1995
Mytilus	F	Ottaviani et al., 1993; 1997; Ottaviani and Franchini, 1995; Stefano et al., 1996; Magazine et al., 1996
Octopus	F	Robertson et al., 1994
Limax	FB	Gelperin, 1994; Gelperin et al., 1996; Moghadam et al., 1995; Moroz et al., 1995
Aplysia	LFB	Jacklet, 1995; Jacklet and Gruhn, 1994; Meulemans et al., 1995; Sawada et al., 1995; Mothet et al., 1996; Moroz et 1996; Robertson et al., 1994
Sepia	LFB	Moroz et al., 1996; Chichery and Chichery, 1994
Helix	LF	Sanchez Alvarez et al., 1994; Pivovarov and Villareal, 1995
Viviparus	LFB	Franchini et al., 1995a; Conte and Ottaviani, 1995
Pleurobranchea,	LB	Moroz and Gillette, 1996; Moroz et al., 1996
Philine, Tritonia, Flabellina, Cadlina, Armina, Coriphella, Doriopsilla, Rossia	L	Moroz et al., 1996
Arthropoda		
Schistocerca	LB	Muller and Bicker, 1994; Bicker and Hahnlein, 1995; Elphick et al., 1993
Rhodnius	B	Ribeiro and Nussenveig, 1993; Yuda et al., 1996
Drosophila	LFB	Muller and Buchner, 1993; Regulski and Tully, 1995; Muller, 1994; Dow et al., 1994
Bombyx	FB	Choi et al., 1995
Triatoma	L	Villar et al., 1994
Pteronemobius	F	Jaffe and Blanco, 1994
Apis	LFB	Muller, 1994; Muller and Hildebrandt, 1995
Cambarellus	L	Talavera et al., 1995
Cancer	LF	Scholz et al., 1996
Nemathelminthes		
Ascaris	L	Bascal et al., 1995
Anellida		
Hirudo	LB	Leake et al., 1995
Plathelminthes		
Hymenolepis	L	Gustafsson et al., 1996
Echinodermata		
Marthasterias	L	Martinez et al., 1994
Coelenterata		
Hydra	FB	Colasanti et al., 1995a
Aiptasia	LFB	Salleo et al., 1996
Protozoa		
Tetrahymena	F	Christensen et al., 1996
Plasmodium	FB	Ghigo et al., 1995

L = Histochemical localization; B = Biochemical demonstration; F = Functional evidence.
For further details *see* Elofsson et al., 1993; Ottaviani et al., 1995; Johansson and Carlberg, 1995.

Table 2
Characterization of Nitric Oxide Synthase in Some Species of Invertebrates.

Source	Transcriptional expression	Ca^{2+} dependence	Calmodulin dependence	Molecular Weight (KDa)[a]	References
Pleurobranchaea	constitutive	–	+		Moroz et al., 1996
Aplysia	constitutive	–	+		Moroz et al., 1996
Sepia	constitutive	+	+		Palumbo et al., 1997
Viviparus[b]	inducible	+			Franchini et al., 1995a
Viviparus[b]	constitutive	+			Franchini et al., 1995a
Apis	constitutive	+	+	150–160	Muller, 1994; Bicker and Hahnlein, 1995
Drosophila	constitutive	+	+	152	Regulski and Tully, 1995; Muller, 1994
Rhodnius	constitutive	+	+	185,130	Ribeiro and Nussenzveig, 1993; Yuda et al., 1996
Bombyx[c]	inducible	+	+		Choi et al., 1995
Bombyx[d]	constitutive	–	+		Choi et al, 1995
Bombyx[d]	constitutive	–	–		Choi et al., 1995
Hydra	constitutive	+	–		Colasanti et al, 1997
Aiptasia	constitutive	+			Salleo et al., 1996
Plasmodium	constitutive	–		< 100	Ghigo et al, 1995
Nocardia	constitutive	+		51.9	Stenger et al., 1996

[a] Monomeric subunit.
[b] Possible presence of other isoforms.
[c] Found in fat bodies.
[d] Found in malpighian tubles.

Roles of NO in Invertebrate Nervous System

Mollusks

Literature data strongly support the presence of NOS activity in mollusk neurons, suggesting a role for NO in the sensorial function associated with feeding and olfaction, and also in synaptic plasticity and learning.

Eloffson et al. (1993) observed a strong NADPH-diaphorase activity in buccal ganglia, osfradia (sensory organs), CNS neurons, and in some peripheral organs. Using an antiserum derived from rat cerebellar NOS-I, an immunocytochemical evidence of NOS is provided in *Lymnaea* (Moroz et al., 1994; 1993). Immunoreactivity was abundant in buccal and perioesophageal ganglia and in the osphradium, suggesting a possible role for NO in the control of both respiratory and feeding activities, and in chemoreception. This view is confirmed in vivo using NOS inhibitors and NO donors.

A thorough study on the olfactory system of *Limax* was performed (Gelperin, 1994; Gelperin et al., 1996; Sanchez Alvarez et al., 1994). In the procerebral lobe, the major central site of odor processing, neurons showed intense NADPH-diaphorase staining. NO scavengers as well as NOS inhibitors were able to reduce the frequency of spontaneous oscillations, these being the basic dynamics of the lobe electric activity. In addition, exogenous NO donors as well as odorant stimulation produced a significant increase in frequency, suggesting that NO may mediate local interactions between afferent fibers from olfactory receptors and efferent neurons. It should be pointed out that NO induces similar effects in the olfactory bulb of mammals (Breer and Shepherd, 1993). Further-

more, CO donors also increase the oscillation frequency in *Limax*. On the basis of these observations and of the robust synaptic plasticity of mollusk olfactory systems, Gelperin suggested that NO and/or CO may have a role in the processing of olfactory stimuli and in the highly developed odor learning ability (Gelperin et al., 1996). Noteworthy are the data concerning CO activity, that may be considered the first report on a biological role of this new signaling molecule in invertebrates.

Jacklet et al. (Jacklet, 1995; Jacklet and Gruhn, 1994) reported that NO may act as a cotransmitter in histaminergic synapses of *Aplysia*, in an identified neuron (C2) that modulates the feeding motor circuit through H1 and H2 receptors. The stimulation of this neuron induces slow excitatory postsynaptic potential in two identified neurons that are insensitive to H1 and H2 histamine-receptor antagonists, but are blocked by NOS inhibitors or by NO scavengers, and mimicked by NO donors. These results suggest that NO works in this case as an orthograde cotransmitter.

NO production in mollusks was directly demonstrated in *Aplysia* both using an NO-sensitive electrode and monitoring nitrite production (Meulemans et al., 1995). NO seemed to control acetylcholine release in buccal and abdominal ganglia, this activity being reduced by NOS inhibitors. The high activity in the chemosensorial apparatus was also confirmed in other species (Moroz and Gillette, 1996). NO implication in chemosensorial activity associated with feeding was further supported by the observation that NO activated neuronal discharges in neurons of the feeding network of *Lymnaea* (Moghadam et al., 1995).

Concerning the transduction mechanisms underlying NOS activity, a link between NO and guanylate cyclase was demonstrated in *Lymnaea* (Elphick et al., 1995). In particular, the authors suggested a role for the NO-cGMP-signaling pathway in mediating chemosensory activation of feeding behavior. These features were confirmed by Sawada et al. (1995) which demonstrated that NO donors and cGMP were able to enhance the Na^+ conductance in *Aplysia* neurons. Furthermore, via a cGMP-activated kinase, NO inhibited an acetylcholine-induced K^+ current in identified *Aplysia* neurons, thus demonstrating a coupling between NO and cGMP. However, other action mechanisms of cGMP could be possible (Mothet et al., 1996). In fact, it has been demonstrated that NO decreases a presynaptic spike-evoked acetylcholine release in identified cholinergic neuroneuronal synapses of the buccal ganglion of *Aplysia*. This inhibition operates through cGMP, but it is not likely to involve protein kinase cGMP-dependent phosphorylation.

Recently, a wide comparative study on NADPH-diaphorase activity and arginine-citrulline conversion has been performed in several mollusk species (Moroz et al., 1995; 1996). The results of these studies provided evidence that a constitutive NOS isoform, Ca^{2+}-independent but calmodulin-dependent, is expressed in the particulate fraction. The susceptibility of the enzyme to the calmodulin inhibitor trifluoperazine (TFP) suggests the intriguing hypothesis that this molluskan NOS may represent a novel NOS isoform. Alternatively, the authors indicated that this molluskan NOS, like the inducible isoforms of the mammals, may constitutively bind calmodulin as a tightly-held cofactor also in the absence of Ca^{2+}. However, it should be pointed out that calmodulin inhibitors are not able to abolish inducible NOS activity, probably because of calmodulin tightly binding to the enzyme.

From a comparative study, a general evolutionary tendency to migration of the nitrergic function from periphery to CNS was hypothesized. Similar differences have been related with the predatory lifestyle in mollusks. In this respect, it was observed that in the herbivorous molluskan species (opisthobranchs), NOS activity is chiefly localized in neurons of peripheral sensorial structures, such as osphradia, lips, or anterior oesophagus, whereas in the predator mollusk *Pleurobranchaea californica*, NOS is preferentially localized in the somata of CNS.

The involvement of NO in learning mechanisms not directly associated with chemoreception has been also proposed. To this effect,

Robertson (1994) observed that intramuscular injections of NOS inhibitors completely block touch-learning in *Octopus*. Moreover, the presence of NOS activity was demonstrated in a cephalopod brain *(Sepia)*, particularly in brain structures that are considered analogs of mammalian cerebellum, thus suggesting NO involvement as a signal molecule in learned motor skills (Chichery and Chichery, 1994). Finally, some authors observed that both NOS and guanylate-cyclase inhibitors can block the long- and short-latent modulation of cholinoreceptor plasticity in gastropod snail neurons (Pivovarov and Egido Vilarreal, 1995).

Arthropods

The first evidence of the presence of NOS activity in arthropod neurons was provided using NADPH-diaphorase histochemistry on the crayfish *Pacifastacus leniusculus* (Elofsson et al., 1993) and *Drosophila* (Muller and Buchner, 1993).

The molecular characterization and cloning of the first invertebrate NOS was obtained in *Drosophila* (Regulski and Tully, 1995). The *Drosophila* NOS gene, dNOS, shows a remarkable conservation between vertebrates and invertebrates. The encoded product is a protein of 152 kDa, with 43% amino acid identity to rat neuronal NOS. The enzyme contains putative binding sites for NADPH, FAD, FMN, and calmodulin. An alternative mRNA splicing exists, which is identical to that known for rat neuronal NOS. When expressed in cell culture, dNOS activity is Ca^{2+}-calmodulin-dependent. These overall data suggest that NOS gene was present in an ancestor common to vertebrates and arthropods; this implies that NOS has existed for at least 600 million yr. Interestingly, the authors reported preliminary data suggesting the presence of other NOS homologs in the *Drosophila* genome.

In the silkworm *Bombyx mori*, Choi et al. (1995) reported the presence of NOS activity in the fat body and in the Malpighian tubles. A lipopolysaccharide-inducible Ca^{2+}-calmodulin-dependent activity, which requires NADPH, FAD, FMN, and tetrahydrobiopterin, represents the main activity of the fat body. In the Malpighian tubles, two types of constitutive NOS were found: one was Ca^{2+}-calmodulin-independent, whereas the other was Ca^{2+}-independent but calmodulin-dependent, thus indicating the presence of new types of NOS in invertebrates.

The full enzymatic characterization of insect NOS was provided both in *Apis* and in *Drosophila* (Muller, 1994). The Ca^{2+}-calmodulin-dependent NOS showed an apparent M_r of 150,000–160,000 and was reported to be very similar to the vertebrate neuronal enzyme. Furthermore, NOS presented a K_m and a V_{max} similar to those reported for mammals. In addition, monomethyl-L-arginine, monomethyl-L-arginine-methylester, or nitro-L-arginine inhibited enzymatic activity. Copurification and inhibition studies suggested that brain insect NOS and NADPH diaphorase are identical, thus confirming that the histochemical method for NADPH diaphorase is suitable for the localization of insect NOS.

Using this histochemical method, the authors observed an evident staining in the chemosensory neuropile, with a strong labeling of neuronal processes but not of neuronal somata. High NOS level in chemosensory neuropiles supported the view that NO has conserved its functional role in chemosensory systems.

Several papers further confirmed the involvement of NO in sensorial mechanisms. NADPH-diaphorase histochemistry, NOS immunoreactivity, and direct biochemical detection demonstrated a Ca^{2+}-stimulated NOS activity in the olfactory neuropile of the antennal lobe of *Schistocerca gregaria* (Muller and Bicker, 1994), in the sensory cerebrum of *Triatoma* (Villar et al., 1994), and in the central nervous system (CNS) of other insects (Bicker and Hahnlein, 1995) and crustaceans (Talavera et al., 1995).

The high activity measured in antennal lobes was confined to the glomeruli (Muller and Hildebrandt, 1995), the sites of synaptic connections between receptor cells, interneurons, and

relay neurons, ideally suited to act as diffusion compartments. Whereas sensor cells of the antenna were stained at a very low level, nonsensory auxiliary cells were markedly stained. Therefore, the latter may have some role in the signal transduction, e.g., in lateral modulation or recruitment of the sensory cells located between the nonsensory ones. Pharmacological experiments demonstrated that the injection of NOS inhibitors did not affect the response to single chemosensorial stimuli, but specifically affected the processing of repetitive stimuli. Injected specimens did not show the normal decrement and disappearance of response following repetitive stimuli. The same effects were induced by injections of guanylate-cyclase inhibitors. These experiments and other ones (Elphick et al., 1993) suggested that the NO/cGMP pathway may be implied in insect chemosensory system, both in the activity of sensory cells (recruitment and/or modulation), and in the CNS with adaptive and/or integrative functions. Thus, a mediation in behavioral plasticity via a cGMP-signaling cascade can be assumed, similar to that described for mollusks.

The hypothesis that in the insect nervous system, NO may play an important role in the processing of olfactory information, is also supported by studies performed on the mushroom body of the honeybee (Bicker 1996). Mushroom bodies are considered important neuropiles for the processing of olfactive information and for the formation of olfactive memory. In these structures, a high density of NOS-expressing neurons was present and the increase of cytoplasmic Ca^{2+}, as provoked by acetylcholine or by other treatments, induced generation of NO by activating a Ca^{2+}-calmodulin-dependent NOS.

As regards the transduction pathway underlying NO activity, the presence of NO-stimulated guanylate cyclase associated with the modulation of olfactory signals was reported in *Schistocerca* brain (Ewer et al., 1994). The mediation of cGMP in arthropod nervous system was also demonstrated in the stomatogastric nervous system of the crab *(Cancer productus)* (Scholz et al., 1996).

NO roles in learning and plasticity, which are not related to chemosensorial functions, are also reported in arthropods by several papers. Beside the report on L-arginine requirement for memory consolidation in the praying mantis (D'Alessio et al., 1982), using NOS inhibitors, NO has been suggested to be involved in the cricket memory consolidation (Jaffe and Blanco, 1994).

Elphick et al. (1996) demonstrated that the optic lobe of the locust *Schistocerca* contains an extensive population of NADPH-diaphorase-positive neurons. The authors proposed that NO is involved in processes such as gain control. In particular, the intense staining found in the dorsal uncrossed bundle (DUB) suggested a possible interaction with a specific neuron, the lobula giant movement detector, responsive to movements of small targets. This neuron is known to exhibit a rapid habituation that is altered after experimental lesions of the DUB. These data suggested an important role for NO in insect vision.

Moreover, as regards a possible involvement of NO in *Drosophila,* Muller (1994) reported that preliminary observations on genetic disruption of NOS function supported the view of some NO implications in developmental and behavioral plasticity.

Also, an involvement of NO in locomotory activity was proposed by Meyer (1994). The author analyzed the distribution of NADPH-diaphorase-positive nerve cells in 10 species of spiders belonging to five families, and on the basis of the localization of stained cells, he suggested a role for NO as intercellular signal also in spiders, with a possible function in high-speed and short-term locomotory action and in the coordination of motor patterns.

The Role of NO in Defense Mechanisms of Invertebrates

The first evidence for a role of NO as an immunocyte-effector molecule in an invertebrate was provided by Radomsky et al. (1991) in the horseshoe crab *(Limulus polyphemus).* Otta-

viani and colleagues (Ottaviani et al., 1993) demonstrated that molluskan immunocytes kill bacteria through two mechanisms, phagocytosis and production of a chemical bactericidal substance, indirectly identified as NO. *E. coli* LPS increases the bactericidal activity and this effect is inhibited by NOS inhibitors.

These data were confirmed by Franchini et al. (1995b) who demonstrated that immunocytes of *Viviparus ater* stained with NADPH-diaphorase reaction and were immunoreactive to anti-NOS mammalian polyclonal antibodies. Treatment with bacterial LPS enhanced NADPH-diaphorase staining. The authors suggested that molluskan immunocytes, in the same way as vertebrate macrophages, respond to bacterial stimulation by inducing iNOS transcription. A better characterization of this mechanism was provided (Franchini et al., 1995a; Conte and Ottaviani, 1995) by directly demonstrating a NOS activity, inducible by stimulation with *E. coli*, in the immunocytes of the mollusk *Viviparus ater*. The enzyme was also partially characterized by the arginine-citrulline assay, both in naive and stimulated cells, with results that were comparable to those described for the mammalian inducible isoform. The enzyme was inhibited by the competitive inhibitor N^G-monomethyl-L-arginine with a K_i of 4.7 μM and the K_m for arginine was 2.5 μM. Moreover, a polyclonal antibody to a rat C-terminal decapeptide produced a concentration-dependent inhibition of mollusk enzyme. Ca^{2+} deprivation induced 30% inhibition of NOS activity. Based on this observation, the authors suggested that mollusk hemocyte NOS may be similar to hepatocyte mammalian NOS, which is considered to be a primitive, nonspecific, widely diffused, defense system. It is also possible that the low inhibition induced by the Ca^{2+} removal is caused by the presence of two different isoforms of the enzyme: a Ca^{2+}-dependent NOS and a Ca^{2+}-independent one. This view is also supported by the observation that LPS stimulation gave a two- to threefold rise in total enzyme activity, whereas in mammalian macrophages a much greater increase has been observed. This can be explained by the presence of two or more isoforms of the enzyme. It is also possible that, coming from a nonsterile wild environment, snails were in a partially stimulated condition; however, it is also possible to speculate that LPS may not be the most effective inducer. NOS activity was present both in the particulate fraction (24%) and in the supernatant (76%). Only the supernatant activity resulted to be stimulated by LPS. NOS activity of hemocytes was also induced by cytokines such as IL-1α, IL-2, and TNF-α.

Other papers analyzed the modulation of NOS activity in the immune system of mollusks (Ottaviani et al., 1995; 1997; Ottaviani and Franchini, 1995; Stefano et al., 1996). In molluskan hemocytes, a stereospecific binding of anandamide, the endogenous ligand of cannabinoid receptors, was found. Treatment with anandamide induced NO release and cell rounding in immunocytes, as well as in human monocytes and microglia. The same effects were induced by morphine operating through μ_3 opiate receptors, and by the NO donor, sodium nitroprusside (Magazine et al., 1996). Both NO release and cell rounding were blocked by a pretreatment with NOS inhibitors. It is important to remember that immunocyte rounding is considered a sign of inactivation that is generally coupled with the repression of iNOS gene activity. In this case it seems possible that the rapid release of receptor-stimulation-induced NO, possibly via Ca^{2+} channels, may be responsible for the repression of iNOS expression (Griscavage et al., 1993). This situation has also been reported for microglial cells (Colasanti et al., 1995b). Taken together, these data support the role of cannabinoid receptors and morphine μ_3 receptors in the modulation of inflammatory and immune responses and the evolutionary conservation of this role.

Liu et al. (1996) also observed that morphine inhibited *Mytilus* microglial motility and was antagonized by NOS inhibitors, suggesting that the opiate was working by a NO-mediated process. This view is confirmed by the observation that microglia constitutively produced NO, NOS inhibitors reduced NO production,

and morphine enhanced NO levels. Opioid peptides on the contrary did not stimulate NO production, thus suggesting that morphine activity was mediated by μ_3 receptors. These data indicate that the NO-morphine relationship known for mammals is also operative in mollusks and that morphine downregulates the activation state of invertebrate microglia. Along with the observations reported by several authors on the role and modulation of NO in the immunocyte-microglial cell lineage of invertebrates, this work supports the close relationship with the monocyte-microglial lineage of mammals.

Other Physiological Roles of NO in Invertebrates

NO and Development

NADPH-diaphorase activity was studied during gangliogenesis and metamorphosis in the gastropod *Ilyanassa obsoleta* (Lin and Leise, 1996). The results provided evidence of the presence of the enzymatic activity in larvae and juveniles and of conspicuous changes during development.

More recently, Palumbo et al. (1997) reported the presence of a Ca^{2+}-dependent NOS and of NMDA receptors in the ink gland of the cattlefish *Sepia officinalis* and proposed a possible role for NO in the maturation and activity of melanin-producing cells.

Developmental changes of NOS activity were also reported in the Malpighian tubles of the silkworm *Bombyx mori* (Choi et al., 1995) with a dramatic increase at the end of the last instar period. These results suggest that NO may play some role during insect development.

A developmental role of NO was also supported by Truman et al. (1996). The authors studied the changes in NO sensitivity, evaluated as cGMP production, during neuronal development in the grasshopper. In most cases, NO sensitivity appeared as a developing neuron switches from axonal outgrowth to maturation and synaptogenesis, to disappear when synaptogenesis is completed. NO sensitivity also appeared in some mature neurons when they underwent synaptic rearrangement.

A thorough analysis of the developmental roles of NO in insects was performed by Kuzin et al. (1996). This paper shows that NO is involved in the control of the size of body structures during *Drosophila* development. The authors found that NOS was expressed at high levels in developing imaginal discs. Inhibition of NOS by local injection of specific inhibitors in larvae caused enhancement of DNA synthesis and hypertrophy of related organs and their segments in adult flies, whereas ectopic expression of NOS in larvae had the opposite effect. Blocking apoptosis unmasked surplus cell proliferation and emphasized the effects of NOS inhibitors also in structures such as eye imaginal discs, where the inhibitors alone have no effects. These results indicate that NO acts as an antiproliferative agent during development, by controlling the balance between cell proliferation and cell differentiation, when considering that the transformation of imaginal precursors in adult structures involves the transition from cell proliferation to cell differentiation. It is important to note that during *Drosophila* development there is a gradual and spatially specific accumulation of NADPH-diaphorase activity in imaginal discs. It is also of interest to speculate how general such a role of NO in development may be. In most cases, the pattern of NOS distribution differs in developing organisms from that of adult organisms. Furthermore, transient elevations of NOS expression during development have been reported, correlated with periods of cessation of cell proliferation. High expression of NOS is also observed during regenerative phenomena, when cessation of the cell division becomes important to avoid unregulated growth. It is interesting to remember that mutant mice with altered NOS expression developed hypertrophy of the stomach that expanded to many times its normal size. NO as a freely diffusible signal molecule, with no need for specific membrane receptors or systems for secretion, is well suited for this

important role in controlling cell proliferation in a coordinate manner. When a group of neighboring proliferating cells produce this diffusible inhibitor, they can share the signal molecules and, when the group reaches a certain size and shape, cell proliferation will cease. In this way, NO may play an important role in controlling the proliferative-differentiative patterns of developing structures.

NO in Blood Sucking Arthropods

A peculiar use of NO was found in *Rhodnius prolixus,* the hematophagus insect vector of Chagas' disease, which produces a salivary vasodilatator with the general properties of NO (Ribeiro et al., 1990).

Ribeiro et al. (1993) demonstrated the presence in *Rhodnius* of a salivary heme protein, nitrophorin, capable of reversible NO binding and responsible for the deep cherry color of the salivary gland. The binding to this Fe(III) heme protein was reversible and dilution at neutral pH promoted NO release. The low binding affinity and fast dissociation kinetics of NO suggested that this protein may act as an NO carrier that helps *Rhodnius* feed on blood.

An NO-carrying hemoprotein was found by Valenzuela et al. (1995) also in *Cimex lectularius.* Considering that *Rhodnius* and *Cimex,* belonging to different hemipteran families, independently evolved to blood feeding, the presence of hemoproteins with similar functions may be a case of convergent evolution.

An NOS activity has been found in the salivary gland of *Rhodnius* (Ribeiro and Nussenzveig, 1993). The enzyme is inhibited by arginine analogs and is dependent on Ca^{2+}, calmodulin, NADPH, FAD, and tetrahydrobiopterin. Molecular sieving indicates a molecular weight of 185 kDa. Based on these results, the authors suggest a similarity with vertebrate constitutive NOS.

From the consideration that imidazole compounds such as histamine can interact with Fe(III) heme proteins, Ribeiro and Walker (1994) investigated the interactions of *Rhodnius* nitrophorins with imidazole compounds. They demonstrated that both imidazole and histamine, but not histidine, bind nitrophorin. This binding leads to displacement of bound NO, as demonstrated by spectral changes and nitrite production. Nitrophorin in this way acts as an antihistaminic substance, as demonstrated by the inhibition of histamine-induced contractures of guinea pig ileum. The authors concluded that the histamine found at the site of the feeding of the hematophagous insect, can be scavenged by the nitrosyl nitrophorins, thus preventing burning pain and itching induced by the bite. Nitrophorins in turn will release the vasodilatatory and platelet-inhibiting NO to counteract the host hemostatic response. By preventing defensive behavioral response of the host and by inducing vasodilation and antiplatelet activity, this complex action of nitrosyl nitrophorins will be to the advantage of the survival of the insect.

NOS from *Rhodnius prolixus* has been cloned, expressed, and characterized by Yuda el al. (1996). The enzyme was soluble, Ca^{2+}-calmodulin-dependent, and differed from mammalian neuronal and endothelial NOS in that it lacked a large N-terminal domain and an N-terminal myristylation sequence. The protein migrated at 130 kDa on SDS-Polyacrylamide gel electrophoresis.

Further studies (Champagne et al., 1995) demonstrated the presence of four distinct nitrophorins, defined NP1–NP4 in decreasing order as per their relative abundance in the glands. Amino acid composition, physical properties, and amino-terminal sequences have been described. NP1, the most abundant, was cloned and its sequence determined. The four proteins (MW approx 20 kDa) were found to be closely related and showed partial homology with invertebrate hemoglobin in being single domain, single subunit molecules with a single heme-binding site. The significance of multiple nitrophorins is unclear, but the four proteins differ from one another in their NO binding and release kinetics, and when combined, they may deliver NO to a greater length of the blood vessel.

NO in Other Invertebrates

Platyhelminthes

Whereas in the first studies (Elofsson et al., 1993) no NOS activity was found in flatworms, Gustaffson el al. in 1996 reported the presence of NADPH-diaphorase reaction in the nervous system of the parasite tapeworm *Hymenolepis diminuta.* They found both CNS and peripheral nervous system (PNS) positive cells. Both cell somata and fibers were stained. Intense staining was found in the motor neuron commissures, indicating a potential role in neuromuscular junctions. NADPH-diaphorase-positive fibers were seen to penetrate the tegument reaching the exterior. These structures can be interpreted as sensory-nerve processes, thus suggesting a role for NO in sensorial functions also in this flatworm.

Annellida

Elofsson et al. (1993) found NADPH-diaphorase staining in the CNS of *Lumbricus* and of *Haemopis.* Leake (Leake and Moroz, 1996; Leake et al., 1995) added evidence of a high activity in the buccal neurons of the leech *Hirudo medicinalis* by studying NADPH-diaphorase staining, citrulline production, NO release, and effects of NOS inhibitors. The strong activity found in sensory neurons suggested that in the anellids NO may also be associated with sensory functions.

Nematodes

As for other low invertebrates, the first studies in nematodes reported negative results (Elofsson et al., 1993). More recently, in *Ascaris suum,* Bascal (Bascal et al., 1995) has described NADPH-diaphorase staining in CNS neurons involved in motility and sensory functions.

Echinoderms

In the starfish *Marthasterias glacialis* (Martinez et al., 1994; Martinez, 1995) NOS immunoreactivity was detected with antibodies specific for the rat neuronal enzyme, but not with antibodies against endothelial or macrophage NOS. The highest reactivity was found in the basiepithelial plexus, which is in direct contact with the flagellated cells of the epithelium. This localization suggested that NO might modulate the activity of flagellated cells, in a way similar to that described for the ciliary beat of airway epithelial cells (Jain et al., 1993). Furthermore, the basiepithelial plexus is proximal to the muscle sheet of the digestive wall, and intense NOS positivity has been found in the piloric stomach. The authors suggested that NO could be involved in muscle relaxation related to the stomach eversion, a feature unique to asteroid echinoderms. This phenomenon is related to the typical extraoral feeding, whereby the everted stomach is inserted between the shells of the bivalve mollusk prey.

NO in Coelenterates

More recently, we have observed for the first time that, surprisingly, the NO-cGMP pathway is present in the freshwater coelenterate *Hydra* (Colasanti et al., 1995a), the most primitive organism possessing a nervous system. Hydra is a sessile predator whose tentacles are armed with the characteristic stinging capsules of the coelenterates, called nematocysts. When a prey accidentally touches a tentacle, a typical feeding response is activated. Hydra feeding response, which can be considered the most primitive olfactory-like activity present in a multicellular organism, is a complex behavioral phenomenon consisting of tentacle writhing and mouth opening. Loomis (1955) determined that the reduced glutathione (GSH) outflow from the prey when pierced by tentacle nematocysts is the physiological activator of hydra feeding response.

We observed (Colasanti et al., 1997) that hydra constitutively express a NADPH-Ca^{2+}-dependent NOS activity. Interestingly, hydra NOS appears to be calmodulin-independent. It is very intriguing to note that a calmodulin-independent/Ca^{2+}-dependent NOS has been

already described in the catfish taste organ (Huque and Brand, 1994) as well as in rat neutrophils (Yoi et al., 1991). Although no evidence is available concerning the molecular evolution of NOS, our results seem to suggest the hypothesis that the primitive NOS isoform as appearing throughout evolution may be a calmodulin-independent isoform. Hydra are able to release basal levels of NO that is enhanced both in the presence of GSH, the chemical activator of hydra feeding response, and after contact with a prey, i.e., a physiological stimulus of hydra feeding response. Initially GSH is likely to induce the feeding response through mediators such as cAMP, IP_3 and/or Ca^{2+}, whereas successively, an increase of GSH-induced Ca^{2+} levels may be responsible for NO production, which in turn elicits the recruitment of neighboring tentacles. This view is supported by the inhibition of tentacle-recruiting provoked by NOS inhibitors and by the induction of tentacle movements by NO donors. On a longer time scale, elevated NO-induced cGMP levels are able to trigger inhibition of GSH-induced feeding response (Colasanti et al., 1995a), presumably via cGMP-activated protein kinases. Taken together with data in the literature, our results are consistent with those reported for the mammalian olfactory system (Breer and Shepherd, 1993). In the latter, in fact, the rapid and transient generation of pulses of cAMP and/or IP_3 are considered the primary reaction in olfactory signal transduction. However, high doses of odorant elicit a delayed and sustained elevation of Ca^{2+} that is sufficient to initiate NO formation. NO is thought to induce the recruitment of neighboring cilia. Finally, the rise in NO-induced cGMP levels is supposed to trigger molecular mechanisms leading to olfactory inhibition (e.g., adaptation processes).

Another interesting action of NO in coelenterates has been recently demonstrated by Salleo et al. (1996). Their data demonstrated that the activation of acontial nematocytes is triggered by a release of NO by the surrounding cells. Indeed, by studying the actinia *Aiptasia diaphana,* these authors found an NOS activity in the acontial tissues. The activity was inhibited by an arginine analog and by Ca^{2+} chelation with EGTA. Staining for NADPH-diaphorase suggested that NOS activity is localized in the supporting cells surrounding the nematocytes. The discharge of nematocytes, normally induced by high K^+, is abolished by NOS inhibitors and restored by excess L-arginine. Direct measurements on K^+-induced discharging nematocytes confirmed that NO is released by stimulated acontia. Both *in situ* and isolated nematocytes discharged when perfused with an aqueous NO solution. These data confirm the observations reported by Colasanti et al. (Colasanti et al., 1995a; 1997) in *Hydra,* showing how far in the evolution NO has played a role in high-speed and short-range communication and coordination.

Protozoa

The production of NO in protozoa is supported by Christienses et al. (1996). The ciliate *Tetrahymena thermophila* produces a survival/growth insulin-like factor which, on the basis of indirect experiments, stimulates an NO-dependent guanylate cyclase. This production is responsible for supporting cell survival and switching the cells in their proliferative mode.

A direct demonstration of NO production in protozoa is reported by Ghigo et al. (1995). The authors provided evidence that human red blood cells infected by *Plasmodium falciparum* synthesize NO (measured as citrulline and nitrite production). This NOS activity is Ca^{2+}-independent, shows an apparent molecular mass < 100 kDa, and is absent in noninfected red-blood cells. This suggests that the parasite expresses an NOS isoform different from those present in mammalian cells. Infected red-blood cells also produce a soluble factor able to induce NOS in normal human endothelial cells.

Interestingly, the saliva of some blood-sucking insects, vectors of leishmaniasis, was demonstrated to possess inhibitory activity on NOS induction of host macrophages (Hall and Titus, 1995), and NO seemed to be re-

quired for the resolution of acute leishmaniasis in resistant animals (Stenger et al., 1996). Bearing in mind the observations of Ribeiro et al. (1990; 1993) on *Rhodnius* saliva and the known ability of NO to inhibit iNOS induction (Griscavage et al., 1993; Colasanti et al., 1995b), speculations on some reciprocal advantage between vector and parasite in leishmaniasis are possible.

NO in Prokaryotic Cells

The presence and characterization of an NOS was reported in *Nocardia* (Chen and Rosazza, 1995). The enzyme is Ca^{2+}-NADPH-dependent, exists as a homodimer, with a molecular weight of 51,900 Da for the monomeric protein. An N-terminal 15-amino-acid sequence was determined showing it to be different from known mammalian NOSs.

General Remarks

The survey of literature data on the presence of NO and its possible roles among in vertebrates demonstrates the widespread diffusion of this signaling molecule throughout the animal kingdom, from mammals down to coelenterates and even to prokaryotic cells. Concerning plants, very few data are available, with a single report of the presence of a putative NOS activity in *Lupinus albus* (Cueto et al., 1996).

The study of the nitrergic system in organisms other than mammals gave the interesting result of the existence of new NOS isoforms, different from those known for higher organisms. In fact the presence both of inducible Ca^{2+}-dependent enzymes and of Ca^{2+}-non-calmodulin or calmodulin-non-Ca^{2+}-dependent forms is reported. The amino acid sequence and molecular weight for multicellular animal organisms show a good degree of conservation, whereas both in protozoa and in prokaryotic cells, the enzyme seems to be different from other known forms.

As far as functional roles are concerned, in invertebrates the main roles described for mammals are found, going from neurotransmission or neuromodulation, to smooth cell relaxation, tubular secretion (Dow et al., 1994) and defense. In addition new functions have been described, mainly in relation to the control of cell proliferation and differentiation, in which NO seems to be a well-suited messenger for this important role. It is probable that incoming research will demonstrate similar functions also for mammals.

Striking is the role played by the nitrergic pathway in the sensory functions, from coelenterates up to mammals, mainly in olfactory-like systems. In this regard, NO is found to be involved both in the diffusion of signals and in adaptive mechanisms as early as in *Hydra* (Colasanti et al., 1995a; 1997), with a conservation of this role in all the species studied in this concern. Of interest is the hypothesis (Moroz et al., 1996; Moroz and Gillette, 1995) of a general evolutionary tendency to migration of the nitrergic function from periphery to CNS. Indeed, in *Hydra* the production of NO seems to be localized in the tentacles, structures directly implied in chemoreception, possibly in non-nervous cells, and both recruiting and adaptation functions operate in tentacles. In higher forms, parallel to the centralization of nervous system first appearing in flatworms, a clear neuronal localization of NOS becomes evident, and finally specialized structures, such as osphradia, are found in mollusks, with a specific NOS activity more than 10-fold higher than mammalian cerebellum. This tendency culminates in mammalian olfactory system, in which peripheral sensory structures have lost NOS activity, now restricted to CNS.

As a general conclusion, of interest are the suggestions (Moncada and Martin, 1993; Feelisch and Martin, 1995; Anbar, 1995; Johansson and Carlberg, 1995) on the early roles of NO in the evolution. NO, originally generated in the primitive atmosphere of our planet, because of its high reactivity, its possible protective action against oxidative damage, and its capability to diffuse through cytoplasm and membranes,

could well have played important roles in the first living forms. In such a way, the cells that first developed the ability to produce such a molecule on their own, well suited to act as a simple signaling system, could have obtained an important selective advantage.

It is thus possible to speculate that NO and other molecules such as CO may have been the first biological signaling molecules, and that future research on the biological roles of these substances in lower living forms will provide important evidence for the understanding of the evolution of signaling systems.

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