Review

Nitric Oxide: An Attractive Signaling Molecule

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I. Introduction

The free radical nitric oxide (NO) is the lowest molecular weight of any known bioactive mammalian cell secretory product. It is an unusual mediator because it is a gas with no known storage mechanism. NO is not stored in synaptic vesicles and does not act on typical receptors on synaptic membranes. Dissolved in the cellular fluids, it can cross cell membranes easily by diffusion. It is highly reactive and extremely labile, so a biological half-life is in the range of only a few seconds, and it is oxidized to stable nitrite (NO_2^-) and nitrate (NO_3^-) [59]. It disappears within moments of its production, thereby obviating a control of its action by release or uptake mechanisms. Therefore, NO action is very transient and directly controlled by the generation of NO. Thus, NO seems to be a novel type of neuronal messenger.

NO is an important messenger molecule having a wider array of functions in many biological systems. It is accounted for endothelial-derived relaxing activity in blood vessels, functioning as a neurotransmitter in the central (CNS) and peripheral (PNS) nervous system and mediating cytotoxic actions of macrophages [10, 57, 64, 69, 88]. Therefore NO is synthesized within mammalian cardiovascular, neural and immune systems. NO dilates all types of blood vessels by stimulating soluble guanylate cyclase and increasing cGMP in smooth muscle cells [44, 69]. In the PNS NO acts as a neurotransmitter of non-adrenergic non-cholinergic (NANC) nerves, regulating relaxation of the gastrointestinal tract [17, 36] and penile erection [46]. In the brain, especially in the cerebellum, NO mediates the ability of the excitatory neurotransmitters glutamate to stimulate cGMP levels via N-methyl-D-aspartate (NMDA) receptors [9, 33, 35]. NO may participate in two forms of long-term synaptic modulation, long-term potentiation in the hippocampus [79, 91] and long-term depression in the cerebellum [80]. NO contributes to the antimicrobial activity of macrophages, par-

ticipating in the destruction of microbial pathogens and tumor cells [40, 67]. Yet NO plays an important role in many parts of the body. During development, NO may influence synaptic pruning, apoptosis and the establishment of the columnar organization of the cortex [24, 32]. Most recent, it is announced that endogenous NO can be a signal for the differentiation of granule cells and Bergmann glia in cerebellar cortical development [84]. Also, NO can cause genomic alterations, through the deaminating of DNA [89]. This mechanism may contribute to the incidence of deamination-related genetic disease and cancer. Thus, NO has a spectrum of effects ranging from physiological control to pathological cytotoxicity. Therefore, NO proves to be a versatile signaling molecule regulating a variety of cellular functions [64, 67].

This review presents a summary of recent information on the mechanisms of NO synthesis and an overview of biological effects of NO.

II. Biosynthesis of NO

NO is formed from the terminal guanido nitrogen atoms of L-arginine through the action of NO synthase (NOS, EC) [4, 10, 11, 39], with the stoichiometric production of L-citrulline [45, 50, 54]. In addition to its substrates L-arginine and O_2 , NOS requires nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin ($\text{H}_4\text{biopterin}$) as cofactors in order to produce citrulline and NO. The conversion of arginine to NO is represented in two steps. The initial step is hydroxylation of L-arginine to generate N^w -hydroxy-L-arginine through the direct oxidation of 1 mol of NADPH by NOS. This step represents the initial two-electron oxidation of L-arginine. Next step is generation of NO and L-citrulline from N^w -hydroxy-L-arginine by oxidation of 0.5 mol of NADPH and $\text{H}_4\text{biopterin}$ [83]. L-citrulline is the co-product, and its ureido oxygen is derived from O_2 .

Biosynthesis of NO from L-arginine represents a five-electron oxidation in both steps. Alternatively, L-arginine can be metabolized by the enzyme arginase to yield ornithine and urea. NOS is inhibited by L-arginine analog with the competitive blockade of the active site. NOS isoforms display modest differences in their sensitivity to various arginine analogs.

Isoenzymes of NOS

There appear to be at least three forms of NOS: endothelial, neuronal, and macrophage isoenzymes. These isoenzymes have now been purified, cloned and expressed [14, 29, 55], and are distinct in antigenicity, NOS appears to be a highly regulated enzyme.

Endothelial NOS and neuronal NOS enzymes are constitutively expressed, requiring calcium ions and calmodulin for their activation. They are stoichiometrically phosphorylated by cyclic AMP-dependent protein kinase, protein kinase C and calcium/calmodulin-dependent protein kinase II, which are important phosphorylating enzymes that regulate cellular responsiveness to hormones, neurotransmitters and growth factors [9, 45]. Therefore, the constitutive NOS participates in signal transduction by generating NO in response to increased intracellular calcium levels.

Macrophage NOS in phagocytic cells may be expressed at low levels under basal conditions. When macrophages are activated by cytokines (eg, interferon- γ , tumor necrosis factor [TNF] and some interleukins) and/or microbial products such as bacterial lipopolysaccharide, NOS is induced in the vascular wall and converts L-arginine into NO. This is induced after several hours of exposure to endotoxins or cytokines. Thus, immunologically induced NOS participates in the destruction of microbial pathogens and tumor cells [40, 67]. This enzyme is dependent on calmodulin, but independent on calcium ion for activity, and requires magnesium ion for maximal activation. Neuronal NOS and macrophage NOS are cytosolic, but endothelial NOS contains both cytosolic and particulate types [28]. There is an important quantitative distinction between constitutive and inducible NO release. Picomolar amounts are released by constitutive NOS while inducible NOS releases nanomoles of NO over long periods.

Despite the differences in regulation and function, the constitutive and inducible NOS are catalytically similar. They have C-terminal binding sites for NADPH; flavins and H₄biopterin as cofactors [11]. They convert L-arginine to NO and L-citrulline with one atom of molecular oxygen binding incorporated into L-citrulline [54], and are inhibited in a specific manner by L-methyl arginine [68]. There are several homogenous but separate NOS genes [57]. Cloning of a complementary DNA for three types of NOS revealed recognition sites for NADPH, FMN, FAD and calmodulin as well as phosphorylation sites [57]. The carboxyl-terminal sequence of NOS displays close homology with that of cytochrome P-450 reductase [14],

another oxidative enzyme characterized by binding sites for NADPH, FAD and FMN in the same polypeptide. Electron transfer between NADPH and two flavins probably has a role as part of NOS catalytic activity. NOS also contains a tightly bound H₄biopterin and iron.

It is likely that many tissues contain distinct isoenzymes for the generation of NO from L-arginine. Some cell types may not only synthesize NOS constitutively, but under certain conditions will synthesize larger amount of NO due to induction of inducible NOS [18]. It has recently been shown that endothelial cells also express a cytokine-inducible NOS. Moreover, neuronal and endothelial NOS, which are generally constitutively expressed, are observed to be regulated by sex hormones, particularly the increased endothelium-dependent relaxation and NO release seen following estrogen treatment or during pregnancy [18].

III. Targets of NO Actions

The main target for the NO action-soluble guanylate cyclase-was characterized at the molecular level. NO activates soluble guanylate cyclase by binding to iron in the heme portion of the active site of the enzyme, altering the enzyme's conformation to augment the catalysis. This is currently thought to be a cardinal mechanism of NO action on vascular smooth muscle relaxant. NO-induced vasodilatation can be mimicked by cGMP, blocked by inhibitors of soluble guanylate cyclase, and potentiated by selective inhibitors of cGMP phosphodiesterase [45, 64]. Hemoglobin can also selectively block the action. Its ability to block is almost carried out by binding of soluble guanylate cyclase in preference to the heme receptor sit on the enzyme [19]. However, the exact mechanisms by which cGMP exerts its biologic effects are not known, although cGMP-dependent protein kinases may be involved.

NO also can be linked to non-heme iron in numerous enzymes, such as iron-sulfur enzymes, and is physiologically related to macrophage microbial function [66, 67]. DNA synthesis is another important target of NO. Macrophage-derived NO on tumor cells inhibits their DNA synthesis through the inhibition of ribonucleotide reductase with the binding to the non-heme iron [53]. Moreover, NO can also cause genomic alterations. NO accelerates base deamination in vitro and causes deamination-induced genetic changes in the living cells [89]. NO activates a cytosolic ADP-ribosyltransferase, but the functional consequences of this ADP-ribosylation are not yet clear [16, 23]. NO is also relevant to superoxide anion through the formation of peroxynitrite anion under pathological conditions [8].

IV. Sites of NO Synthesis

The detailed anatomical localization of the sites of NO synthesis is studied using immunocytochemical stain-

ing with anti-NOS antibody [12]. In the brain, NOS occurs primarily in neurons and also in the endothelium of blood vessels with no glial localizations. NOS in the brain is selectively concentrated in the cerebellum, the olfactory bulb, the supra optic, paraventricular and dorsal raphe nuclei, the superior and inferior colliculi and the dentate gyrus of the hippocampus (Fig. 1). In the PNS, NOS immunoreactivity is evident in the myenteric plexus of the intestinal tract, anococcygeal nerves, and retinal autonomic nerve fibers [12]. In the periphery, NOS is enriched in the endothelium of blood vessels, posterior pituitary gland, adrenal medulla, kidney, lung, pancreas, uterus and stomach [20, 78]. The localization of NOS in neurons of CNS and PNS is confirmed to that of NO action which may regulate cerebral blood flow and mediate long-term potentiation. Electron microscopically neuronal NOS immunoreactivity is not specifically associated with any subcellular organelle or with the plasma membrane

[56]. This localization supports that NO is not stored and released [56].

NO is formed from L-arginine by NOS, with the stoichiometric production of L-citrulline, and activates soluble guanylate cyclase to increase cGMP levels. Therefore, the localization of these NO-related substances, such as L-arginine, L-citrulline and cGMP, may supply additional information on the sites of NO synthesis and the target cells where NO stimulates guanylate cyclase. L-arginine immunoreactivity in the rat brain and spinal cord is evident mainly in astrocytes including Bergmann glial cells in the cerebellum and processes of astrocytes wrapping vascular endothelial cells and smooth muscle [4] (Fig. 2). In the PNS including cochlea, dorsal root and superior cervical ganglia and enteric plexus, L-arginine is enriched in satellite and supporting cells, both of which ensheath neurons as glial cells do so in the CNS [2]. Thus, neurons are surrounded by such an L-arginine

Fig. 1. Immunocytochemical localization of NOS in the accessory olfactory bulb (a) and dorsal raphe (b), supraoptic (c) and paraventricular (d) nuclei. (a) Granule cells were intensely stained. Bars=100 μ m

pool. In peripheral tissues containing paraneurons [30], the localization of L-arginine is also studied [3]. In the tissues containing glia-like cells, L-arginine is concentrated in the glia-like cells surrounding the paraneurons or endocrine cells, that is, in folliculo-stellate cells of adenohypophysis, stellate cells of adrenal medulla and outer phalangeal cells of Corti's organ. In the thyroid gland, parathyroid gland and pancreatic islets of Langerhans, which do not contain glia-like cells, L-arginine is localized in the paraneuron itself [3]. L-citrulline is localized in neurons in both the CNS [70] and PNS [5]. Citrulline-positive neurons have a restricted distribution in the brain. Strongly stained cells are localized in the striatum, dorsal raphe and pedunculopontine nuclei and olfactory bulb (Fig. 3). A few cells are present in the cortex and corpus callosum. These distributions of citrulline immunoreactivity is similar to that of NOS-positive neurons in several brain regions (Fig. 4). Thus, in the CNS the colocalization of L-citrulline and NOS is likely, however, in the PNS the colocalization of these substances is not always true [1]. NO mediates the effects of glutamate in elevating cGMP levels in the cerebellum [9, 35], therefore guanylate cyclase is certainly a target of NO. However, in other brain areas the localization of NOS does not resemble that of guanylate cyclase mapped by immunocytochemistry [65, 78]. Guanylate cyclase is located in NOS-negative neurons, indicating that NO may act in other ways than via guanylate cyclase and/or guanylate cyclase may be regulated by other transmitters besides NO.

The mRNA for NOS and NOS itself are colocalized with neurons enriched in NADPH diaphorase (NADPHD) activity, therefore the identification of NOS was fully performed by NADPHD histochemical reaction [13, 21]. So, this simple histochemical method by the mitroblue

tetrazolium reaction is generally used to show the anatomical localization of neurons generating NO [38]. However, after this the histochemical method was also revealed to visualize other reducing enzymes as well [71]. Because NADPHD is characterized to have an oxidative activity dependent on NADPH [52]. NADPHD activity of rat brain is located mostly in the particulate fraction, whereas most of NOS activity is in the cytosolic fraction [60]. Also, the distribution of NADPHD activity in the brain is different from that of NOS with the biochemical assay. Pretreatment of the fractions with paraformaldehyde virtually abolished the NADPHD activity in the particulate fraction, whereas 40–60% of the NADPHD activity remains intact in the cytosolic fraction. Most NADPHD activity is inactivated during fixation and only some of the NADPHD activity associated with soluble NOS remains intact. The correlation between neuronal NOS and NADPHD in histochemical studies [13, 21, 42] may be accounted for the inactivation of most non-NOS NADPHD activity during the fixation procedure. This should be kept in mind when making a conclusion about the distribution of NOS based on NADPHD staining of tissues.

Neuronal NOS is not confined to neurons but is widely distributed over several non-neuronal cell types and tissues [78]. These includes glia cells, macula densa of kidney, epithelial cells of lung, uterus, stomach, and islets of Langerhans. From these localizations neuronal NOS is thought to be the most widely distributed among isoforms [64], and in addition to its neural functions, regulates secretion and non-vascular smooth muscle function [38].

V. Roles of NO

The widespread cellular localization of NOS and the short half-life and diffusion properties of NO have led to

Fig. 2. Immunocytochemical localization of L-arginine. Bergmann glial cells (a) and astrocytes wrapping vascular endothelial cells in the granule cell layer (b) are intensely stained. P: Purkinje cells, V: blood vessels. Bars = 50 μ m (a), 25 μ m (b).

Fig. 3. Immunocytochemical localization of L-citrulline in the granule cells of the accessory olfactory bulb (a), dorsal raphe nucleus (b) and striatum (c). Bars = 100 μ m

Fig. 4. Colocalization of NOS (a) and L-citrulline (b) positive neurons in the pedunculo pontine tegmental nucleus. Fluorescent staining was accomplished with Texas Red labeled anti-mouse IgG for NOS and FITC labeled anti-rabbit IgG for L-citrulline. Bars = 50 μ m

speculation that NO plays a key role in many biological systems.

In blood vessels, NO mediates endothelium-dependent vasodilation in response to acetylcholine, bradykinin, and other mediators. NO is known to activate soluble guanylate cyclase of the vascular smooth muscle, leading to an increase in the second messenger cGMP and thereby maintaining the basal vascular tone and regulating regional blood flow [44, 69].

In the immune system, NO is produced by activated macrophages and neutrophils as a cytotoxic agent targeting tumor cells and pathogens, and involved in killing and cytostatic action [40, 66, 67]. While a signaling role in macrophages is not ruled out, these cells generate NO as a cytotoxic agent. NO levels also increase in response to shear stress and to mediators of inflammation [44].

In the nervous system NO plays a role as a signaling molecule [34, 81]. In many systems the action of NO is carried out through the activation of soluble guanylate cyclase and the production of cGMP. Therefore, NO is mediated in most biological effects by cGMP as the second messenger. NO appears to influence neurotransmitter release. In several model systems, NOS inhibitors such as nitroarginine blocked the release of neurotransmitters [90]. In brain synaptosomes, the release of neurotransmitter evoked by stimulation of NMDA receptors is blocked by nitroarginine, while release elicited by potassium depolarization is not affected [39]. Presumably glutamate acts at NMDA receptors on NOS neurons to stimulate the formation of NO, which diffuses to adjacent terminals to enhance the neurotransmitter release so that the blockade of NO formation can inhibit the release of transmitters [39].

NO is also implicated as a potential mediator of NMDA-induced neurotoxicity. Glutamate neurotoxicity elicited via NMDA receptors mediates much of the neurotoxicity in cerebral ischemia. Neuronal cell damage due to cerebral ischemia is mediated by NO. Cerebral ischemia induces excessive NMDA receptor activation and subsequent overentry of calcium. Consequently, excessive NO is produced and reacts with superoxide anion to form peroxynitrite anions. These rapidly decompose to yield highly damaging hydroxy radical and nitrogen dioxide, leading to ischemic cerebral damage [8].

NO is thought to also play a role in synaptic plasticity by a diffusible retrograde message, which could spread laterally and potentiate transmission at inactive presynaptic terminals. The concept of the retrograde message is thought to participate in two forms of long-term synaptic modulation [79]. First form is long-term potentiation (LTP), which contributes to the synaptic network of storing memories in mammals [79, 91]. LTP is triggered when a neuron receives several simultaneous signals. The signals trigger NMDA receptors, one of the glutamate receptors, to let calcium ions flow into the cell. The calcium ions cause the synapses that receive the

simultaneous signals to be strengthened. Some of the strengthening is due to an increase in the amount of neurotransmitter released by the sending cell. For example, induction of LTP in the CA1 region of the hippocampus requires calcium ions influx through postsynaptic NMDA receptor channels, and maintenance of LTP involves a presynaptic increase in transmitter release, implying that the postsynaptic cell must carry retrograde messages back to the presynaptic terminals [79]. This feedback is central to some types of learning. In the proposed scheme, a nerve cell received message would send a retrograde messenger back to the sending cell, strengthening the connection between them and contributing to the formation of a long term memory.

Another form of synaptic plasticity is long-term depression (LTD) in the parallel fiber-Purkinje cell synapses in the cerebellum [80]. Conjunctive stimulation of climbing and parallel fibers leads to LTD of the ability of the parallel fiber to activate the Purkinje cell [47]. Since the output of the Purkinje cells is inhibitory, the effect will be increased firing of the Purkinje cells' target neuron. This phenomenon is implicated as the cellular mechanism for cerebellar motor learning. Shibuki and Okada [80] demonstrated that endogenous NO was released after stimulation of climbing fibers, that LTD was blocked by hemoglobin (which strongly binds NO) or L-NG-monomethyl-arginine (an inhibitor of NOS), and that exogenous NO or cGMP could substitute for the climbing fiber stimulation to cause LTD. Diffusible nature of NO enables the induction of synaptic plasticity in the cerebellum. Thus, NO may serve as the retrograde neurotransmitter to enhance synaptic function due to correlated firing of pre- and postsynaptic cells.

In the mammalian retina NADPHD activity was localized histochemically in neurons contributing to the inner plexiform layer, presumably amacrine cells [77]. NOS activity was biochemically demonstrated in rod outer segments of bovine retina [87] and activation of guanylate cyclase by NO affects the dark membrane potential and the light response of frog retinal rods [85]. The L-arginine: NO pathway may regulate the mechanism of gap junctions in horizontal cells [62]. This data indicates a functional role of NO in the signal processing within the retina.

In the PNS, NO mediates relaxation of smooth muscle in the gastrointestinal system and the anococcygeous muscle, depending on the autonomic nerve system or inhibitory NANC nerves [12, 36, 37].

There is increasing evidence that NO is synthesized in the kidney and plays an important role in the regulation of renal hemodynamics and excretory function [76]. Both glomerular and medullary microcirculations in rat kidney are regulated by endogenous NO [15, 74]. NO mediates arterial pressure-related changes in urine flow and sodium excretion and may control tubular reabsorption [22, 75]. The source of NO is the endothelium, macula densa, and mesangial cells in glomeruli [6]. Bradykinin and acetylcholine induce renal vasodilation by increasing NO

synthesis. The blockade of basal NO synthesis was shown to result in decreases of renal blood flow and sodium excretion. The macula densa may modulate tubuloglomerular feedback response through regulating afferent arteriolar contraction by NO release. In the proximal tubule, NO possibly mediates the effects of angiotensin on tubular reabsorption [22, 75]. In the collecting duct, an NO-dependent inhibition of solute transport is suggested. Thus, NO is a highly versatile molecule that regulates a large number of cellular functions.

Despite these reports on the important roles of NO, a serious doubt for its necessity for life is presented. Recently, Huang *et al.* [43] have studied the function of the neuronal NOS enzyme by disrupting and inactivating its gene. They produced a new mutant mouse which lacks the neuronal NOS gene by homologous recombination. The effects of disrupting the neuronal NOS gene during development can be observed in intact animals. NOS catalytic activity is depleted from the mouse brain, and NOS staining is undetectable in central and peripheral neurons. Surprisingly however, these animals appeared normal in most respects. The neurons normally expressing NOS appeared intact, and the mutant NOS mice were viable, fertile, and without evident histopathological abnormalities in the CNS. The stomachs of mutant mice are greatly distended compared to the age-matched control mouse, due to hypertrophy of the circular muscle layer of the stomach and pylorus, presumably due to enteric nervous system dysfunction. Evident effects of disrupting the neuronal NOS gene is only on the gastrointestinal tract. The reason may be that other converging pathways can also play compensatory roles.

VI. Clinical Horizons

NADPHD positive neurons, that is NOS containing neurons, survive neurotoxic insults. NADPHD neurons are selectively resistant to the clinical degenerative loss associated with several diseases such as Huntington's disease [25], and appear to be resistant to ischemic destruction [86] and survive the excitatory neurotoxin-induced destruction of neural tissue [7, 51]. In Huntington's disease up to 95 percent of neurons in the caudate nucleus and striatum degenerate, while virtually all NADPHD neurons are preserved [25]. A significant increase in the activity of this enzyme in these areas is observed in postmortem brain tissue from patients with this disease [25]. In Alzheimer's disease NADPHD neurons are similarly resistant [82]. Neurotoxic destruction by NMDA in primary cerebral culture, a model for stroke, was observed in 90 percent of neurons, whereas NADPHD neurons survive completely. It's not clear why NADPHD neurons survive these neurotoxic insults including stroke, Alzheimer's disease and Huntington's disease. NOS inhibitors selectively prevent glutamatergic neurotoxicity mediated by NMDA receptors in primary brain cultures, indicating that NO secreted by NOS neurons may kill

adjacent neurons [21]. So, NOS activity, which accounts for the NADPHD staining, may be responsible for this resistance.

NO plays a part in several important physiological processes, and may act as a neurotransmitter. Constitutive NOS appears to provide critical homeostatic and regulatory functions in the vascular and nervous systems. Inducible NOS is enhanced by endotoxin and certain cytokines in macrophage. Therefore, impairment of NO synthesis or over production of NO are observed in many circumstances and result in circular diseases. NO is implicated in several pathological states, including septic shock, vascular leak syndrome associated with cytokine therapy, uremia, and diabetes [48, 49, 72]. N^G-monomethyl-L-arginine, an inhibitor of endogenous NO synthesis, is useful for the treatment of hypotension associated with these pathological states. The hypertensive animals showed the reduced endothelium-mediated vasodilatation in arteries and also hypertensive patients showed the impaired responses to endothelium-dependent vasodilators [58]. Hypercholesterolemia and atherosclerosis are also associated with impaired endothelium-mediated vasodilatation *in vitro* and *in vivo*, in animals and humans [27]. Inhaled NO seems to be a selective and effective pulmonary vasodilator in pulmonary hypertension [73]. The treatment of interleukin-2 for patients with malignant melanoma and renal carcinoma causes an increase in the plasma levels of TNF- α and an increase of nitrate (the breakdown product of NO) in plasma and urine [41]. Thus, NO gas and NOS inhibitors are now being used for therapy.

VII. Conclusions

NO is a very attractive and important messenger molecule. An area of research for NO has been rapidly expanding from the discovery of NO. It is a novel type messenger molecule: small molecular weight, highly diffusible, gaseous, and short-lived, so NO can freely pass through the cell membranes and can send messages to adjacent synapses and/or retrograde messages, having surprising characteristics. NO localizes in a variety of different cell types and controls a number of critical physiological processes. Its biological actions range from signal transduction to cell killing. In particular, the induction of neuronal NOS by estrogen and testosterone is likely to have a considerable impact on the understanding of the nervous system. Therapeutic application of NO and NOS inhibitor is also very useful for the patients with pathological states. More expanding of NO roles and an application for therapy is expected.

VIII. References

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